Remarks

Reconsideration of this Application is respectfully requested. Upon entry of the foregoing amendment, claims 48-61 are pending in the application, with claims 48, 51, 52, and 57 being the independent claims. Claims 30-47 are sought to be cancelled without prejudice to or disclaimer of the subject matter therein. New claims 48-61 are sought to be added.

Presentation of claims 48-61 is believed to be consonant with Applicants' previous election of Group III (i.e., methods of treating a disease or condition comprising administrating an effective amount of a lectin). *See* Reply to Restriction Requirement, filed February 27, 2004; and Office Communication mailed January 29, 2004, "Supplemental Election/Restrictions," beginning on page 3. Furthermore, claims 48-61 read on the previously elected species, a conjugate comprising *Erythrina cristagalli* lectin.

Support for these new claims can be found throughout the specification and the previously presented claims. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

I. Written Description Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 39-42 are rejected under 35 U.S.C. § 112, first paragraph. In particular, the examiner has alleged that these claims contain "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the

relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." Office Action, paragraph bridging pages 2-3. As discussed below, this written description-rejection is directed to i) the scope of diseases and conditions treated as well as ii) the scope of conjugates. Applicants respectfully traverse both aspects of the rejection.

A. The Written Description Legal Standard and USPTO Practice

The statutory written description requirement is essentially concerned with whether the specification (including the original claims) describes the claimed invention in sufficient detail that the *ordinarily skilled artisan* can reasonably conclude that the inventor had possession of the claimed invention. *See* M.P.E.P., 8th ed., §2163 (Rev. 2, May 2004). Recently, in *Enzo Biochem Inc. v. Gen-Probe Inc.*, 63 USPQ2d 1609 (Fed. Cir. 2002), the Federal Circuit adopted a portion of the USPTO guidelines regarding the written description requirement. In particular, the adopted portion states that the requirement can be met by "show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . *i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.*" *Id.* at 1613 (emphasis added). Hence, adequate written description of the claims may be shown by coupling the description of the specification with the knowledge available to the skilled artisan at the time of the invention.

Federal courts have also indicated that the Patent Office has the initial burden of demonstrating that an application does not satisfy the written description requirement:

> A description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. See, e.g., *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). The examiner, therefore, must have a reasonable basis to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. [*In re*] *Wertheim*, 541 F.2d [257] at 263, 191 USPQ [90] at 97.

M.P.E.P., 8th ed., §2163.04 (Rev. 2, May 2004). Hence, the Examiner bears the initial burden of setting forth a *prima facie* case that the claims are not adequately described. Moreover, in the context of *original claims*, "[t]here is a *strong presumption* that an adequate written description of the claimed invention is present when the application is filed. *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976) . . . ." M.P.E.P., 8th ed., §2163, "A. Original Claims," (Rev. 2, May 2004) (emphasis added).

B. Description of Diseases and Conditions Treated

1. Alleged Reasons for Written Description Rejection

With respect to the scope of diseases and conditions treated, the Examiner has alleged that

> One of skill in the art would not recognize from the disclosure that the Applicant was in possession of the claimed invention, namely treating any C-fibre neuron associated diseases/conditions (claim 39), nor the distinctly claimed conditions of psoriasis and mucus hypersecretion (claim 42), by an ECL conjugate. The specification does not "clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed" (see *Vas-Cath* at page 1116). Namely, the specification has adequately described the use of ECL conjugates in a method of treating two (2) C-fibre associated diseases or conditions: pain (i.e. analgesic affect in mouse, See Figure 3 and Example 5 and 6) and inflammation (pretreatment stimulated rat paw, See Figure 13 and Example 18). There is no description as to how or whether an ECL conjugate would be able [to] treat the other [] 2 specifically claimed C-fibre neuron diseases/conditions (psoriasis and mucus hypersecretion) of claim 42 or any C-fibre neuron diseases/conditions as contemplated by

> claim 39. One of skill in the art would not recognize from the disclosure that the Applicant was in possession of a method of treating any C-fibre neuron associated diseases/conditions; other than pain and inflammation, using an ECL conjugate.

Office Action, paragraph bridging pages 3-4. Applicants respectfully disagree.

2. A Prima Facie Case for a Written Description Rejection Has Not Been Set Forth

The Examiner has not set forth a *prima facie* case that the claims are not supported by an adequate written description. First, it is noted that Applicants' claims 39-42 find complete support in original claims 23-30 on pages 42-43 of the specification. Moreover, a description of C-fibre activity and the relationship of that activity to diseases or conditions is provided throughout the specification. *See, e.g.,* pages 1-4. It is noted that psoriasis and mucus hypersecretion are specifically described as C-fibre related diseases or conditions. *See e.g.* page 2, lines 10-31, and page 4, lines 15-17. Finally, Applicants' specification describes a method of treating these diseases or conditions by administering to a patient an agent that inhibits that activity. *See e.g.* page 4, lines 24-28, and the examples in pages 18-39. As such, Applicants' claims are presumptively adequately described for the skilled artisan.

However, the Examiner has not provided *any* evidence why such a person would not recognize in Applicants' disclosure a description of the invention defined by the claims. Such evidence should begin with a description of the skilled artisan and his or her knowledge to provide the proper perspective and framework from which to evaluate the adequacy of Applicants' written description. The Examiner entirely fails to define the skilled artisan or the knowledge generally known to him or her at the time the application was filed.

Here, the skilled artisan would necessarily have general knowledge pertaining to C-fibre neurons and their activity in the context of patient diseases or conditions. But the written description rejection was not made from the perspective of such a skilled artisan. Rather, the Examiner's allegations focus solely upon alleged limitations of Applicants' exemplified teachings irrespective of the skilled artisan's familiarity with C-fibre neuron associated diseases or conditions. Applicants respectfully assert that the foundation of any *prima facie* case for lack of written description *must begin from the perspective of the skilled artisan.* Since the rejection was not provided from this perspective, its allegations are baseless and should be withdrawn.

3. The Skilled Artisan Understands That C-Fibre Neuron Activity Related Diseases or Conditions Arise From Stimulation of C-Fibre Neurons

Notwithstanding that a *prima facie* case for a written description rejection has not been set forth, Applicants provide the following statements regarding the perspective of the skilled artisan for the convenience of the examiner.

Prior to the present application, the skilled artisan understood that stimulation of C-fibre neuron activity resulted in the undesirable release of neurotransmitters or neuromodulators, such as substance P. *See* Specification, page 1, lines 21-32. The link between this undesirable stimulation and corresponding medical diseases or conditions was also known. *See e.g.* pages 1-4. For example, as explained towards the bottom of page 35 of the present specification, a skilled person would appreciate that the present invention has application in the treatment of sunburn and psoriasis in the skin, migraine, asthma and rheumatoid arthritis, all of which involve neurogenic inflammation, i.e., stimulation of neurons whereby neuropeptides (such as substance P) are released.

Hence, the skilled artisan appreciates the panoply of diseases or conditions associated with C-fibre neurons. Moreover, the skilled artisan appreciates that these diseases or conditions arise from the stimulation of C-fibre neurons.

In addition, publications predating or contemporeaneous with the captioned application clearly establish that stimulation of C-fibre neurons (also termed C-fibre nociceptors (Specification page 1, line 21)) was associated with medical diseases or conditions. For example, a brief review of chapter titles and opening pages of *Neurobiology of Nociceptors,* Belmonte, C. and Cervero, F., eds., Oxford University Press, New York, chapters 14-18 (1996) (Exhibit A) confirm the following conditions which result from undesirable C-fibre stimulation:

- pain & hyperalgesia;
- cutaneous dysaethesiae (abnormal sensory states, e.g. hyperalgesia and hyperknesis (i.e., itch));
- efferent function (including vasodilation and other aspects of 'neurogenic inflammation', manifest in a range of diseases such as asthma and arthiritis); and
- neuroimmune functions (including the response from inflammatory cells following release of mediators from neuronal source, such as substance P, CGRP & opioids).

In addition, a brief review of *Textbook of Pain*, 4th ed., Wall, P.D. and Melzack, R., eds., Churchill Livingstone, New York, pp. 35-36 (1999) (Exhibit B) reveals the following medical diseases or conditions resulting from undesirable nociceptor effects (i.e., stimulated C-fibre neuron activity):

- neurogenic inflammation;
- neuroimmune functions;
- smooth muscle contraction; and

- glandular secretion from organs (e.g. airways, gastrointestinal tract, and urinary tract).

The link between C-fibre stimulation and psoriasis had also been established in literature prior to the present application. Naukkarinen, A. *et al., J. Invest. Dermatol., 92*: 126-9 (1989) confirms that "substance P may be an important mediator in the inflammatory processes that contribute either to the initiation or maintenance of a psoriatic lesion." Exhibit C, Abstract, last sentence. The same authors also observed that the "epidermis and dermis of the psoriatic lesions were significantly more densely innervated with neurofilament-positive fibers [i.e., sensory nerves, namely C-fibres] than . . . control skin." *See* Exhibit C. Farber *et al., J. Am. Acad. Dermatol., 14*: 305-311 (1986) indicates that the number of substance P-positive fibres (i.e., C-fibres) were significantly enhanced in psoriatic skin compared to normal skin. *See* Abstract provided as Exhibit D.

The link between C-fibre stimulation and mucus hypersecretion has also been well established in the literature. For example, U.S. Patent No. 6,632,440 confirms that C-fibre release of substance P results in hypersecretion of mucus from mucus-secreting cells. Thus, by reducing the undesirable secretion of neurotransmitters or neuromodulators from C-fibres, conditions such as mucus-hypersecretion (and related conditions such as chronic obstructive pulmonary disease (COPD) and asthma) are treatable.

The unifying and central mechanism of action of these diseases or conditions is stimulated C-fibre activity with concomitant release of neurotransmitters or neuromodulators, such as substance P. The skilled person would readily glean from standard medical text books additional medical disease or conditions resulting from

stimulated C-fibre neuron activity which may therefore be addressed by the present invention.

Hence, the skilled artisan appreciated at the time the captioned application was filed, that a class of medical diseases or conditions are associated with C-fibre neurons. Moreover, the skilled artisan would recognize that these diseases or conditions arise from the stimulation of the C-fibre neurons. Hence, as evidenced by the following section, the skilled artisan would appreciate that an agent capable of inhibiting such stimulation could effectively be used to treat *any* of the diseases or conditions associated with that activity (i.e., associated with C-fibre activity). As described below, Applicants' specification adequately describes for the skilled artisan agents and methods for inhibiting C-fibre neuron activity.

4. The Specification Adequately Describes Treating Any C-Fibre Neuron Activity Disease or Condition by Inhibiting C-Fibre Neuron Activity

Example 14 and Figure 10 confirm that ECL (a galactosyl-binding lectin) is capable of inhibiting neurotransmitter or neuromodulator release (here, substance P) from C-fibres when administered at high concentrations. In this regard, at concentrations in excess of 10 μg/ml, positive values indicate that substance P release is inhibited. It is this inhibition of neurotransmitter or neuromodulator release from C-fibres that underpins the inhibiting aspect of the present invention. Similarly, Example 15 and Figure 11 confirm that an ECL conjugate is capable of inhibiting neurotransmitter or neuromodulator release (here, substance P) from C-fibres when administered at high concentrations.

Example 5 and Figure 2 demonstrate the analgesic activity (i.e., pain treating ability) of the lectin ECL. This effect is achieved through the inhibition of neurotransmitter or neuromodulator release from C-fibres as shown in Figure 10. Additional data are illustrated in Figure 14, in which a high concentration of lectin (e.g., greater than 1 μg) results in a dose-dependent *in vitro* analgesic effect. This is achieved through inhibition of neurotransmitter or neuromodulator release from C-fibres (see Figure 10).

Example 7 describes the use of WGA (i.e., a glucosyl-binding lectin). As described immediately above with reference to Example 5 and Figure 2, WGA lectin (like ECL) is also capable of demonstrating an analgesic effect. Again, this is achieved by inhibiting release of neurotransmitter or neuromodulator from C-fibre as described above for ECL.

Example 8 and Figure 5 demonstrate the use of the lectin IB_4 (i.e., a galactosyl-binding lectin). In more detail Figure 5 confirms that this lectin is capable of demonstrating an analgesic effect and, consistent with Figure 10, this is achieved through the inhibition of neurotransmitter or neuromodulator release from C-fibres.

Example 18 and Figure 13 demonstrate that ECL has an anti-inflammatory effect. Again, as discussed above, this effect is achieved through the inhibition of neurotransmitter or neuromodulator release from C-fibres.

Example 16 and Figure 12 demonstrate that application of ECL markedly reduced C-fibre response, indicating an overall inhibition in C-fibre activity.

Based on the above comments and Examples, a skilled person would understand that the inhibiting aspect of the present invention is achieved through binding of a lectin

to a galactosyl or glucosyl residue, which binding event results in the inhibition of neurotransmitter or neuromodulator release from C-fibres. The skilled artisan would appreciate from Applicants' specification that the principle that underlies this inhibiting aspect of the present invention is the inhibition of neurotransmitter or neuromodulator release from C-fibres.

Thus, in view of the underpinning principle (i.e., inhibition of neurotransmitter or neuromodulator release) the skilled artisan would readily appreciate that the present invention would have application in the treatment of any disease or condition resulting from the undesirable stimulation of C-fibre neuron activity.

5. The Specification Also Adequately Describes Stimulating C-Fibre Neuron Activity

In contrast to the above-described inhibition of C-fibre activity, the present invention may separately achieve stimulation of C-fibre neuron activity. In this regard, the simple determining factor is that of lectin (or lectin conjugate) *dosage*.

Thus, turning to Figure 10 of the present specification, at low dosages (e.g., less than approximately 10 μg), the inhibitions of the neurotransmitter or neuromodulator release is a negative value. This signifies stimulation of the release of neurotransmitter or neuromodulator from C-fibres. In this regard, Figure 10 illustrates the stimulating effect of lectin. Figure 11 confirms a similar trend in stimulation (i.e., negative values along the y-axis) when the lectin is present in the form of a conjugate (i.e., when the lectin is conjugated to a companion peptide or protein).

Thus, the only difference between the inhibition and stimulation aspects of the present invention is that of dosage, and a skilled person would readily accept Figures 10

and 11 of the present specification as adequate demonstration that the lectins and lectin conjugates recited in the present claims are capable of exerting the defined stimulating C-fibre neuron activity.

C. Description of Conjugates

1. Alleged Reasons for Written Description Rejection

With respect to the scope of conjugates, the Examiner has alleged that

> One of skill in the art would not recognize from the disclosure that the Applicant was in possession of the claimed invention, namely any ECL "conjugate" for use in the method(s) of the present invention. The specification does not "clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed" (see *Vas-Cath* at page 1116). Namely, the specification has adequately [described] the conjugation of ECL to the clostridial enzyme designated LH_N/A (See Example 3). There is no description for any other compound conjugated to ECL, nor has any other ECL conjugate been tested for use in the methods of the present invention. One of skill in the art would not recognize from the disclosure that the Applicant was in possession of a method of treating any C-fibre neuron associated diseases/conditions; other than pain and inflammation, by any ECL conjugate, other than ECL-LH_N/A.

Office Action, page 4, first full paragraph. Applicants respectfully disagree.

2. A Prima Facie Case for a Written Description Rejection Has Not Been Set Forth

For the reasons given above in section ***I.B.*** and wholly incorporated here, the Examiner has not set forth a *prima facie* case that the claims are not supported by an adequate written description. As with the written description rejection discussed in section ***I.B.*** above, the Examiner has not provided *any* evidence why the skilled artisan would not recognize in Applicants' disclosure a description of the invention defined by the claims. As above, this aspect of the rejection also fails to define the skilled artisan or the knowledge generally known to him or her at the time the application was filed.

Hence, this aspect of the written description rejection is baseless and should be withdrawn.

3. The Skilled Artisan Recognizes Upon Reading Applicants' Specification that C-Fibre Stimulatory or Inhibitory Action is Caused by the Lectin, and Would Accordingly Appreciate that any Conjugates Thereof Can be Used According to the Invention

Notwithstanding that a *prima facie* case for a written description rejection has not been set forth, Applicants provide the following comments regarding what the skilled artisan would recognize upon reading Applicants' specification.

From the examples described above in section ***I.B.4.***, the skilled artisan would appreciate that the active component of the present invention is a lectin. For example, Figure 10 illustrates that low and high concentrations of unconjugated lectin respectively have stimulatory and inhibitory effects on C-fibre neurons. This trend is paralleled in Figure 11 which illustrates that low and high concentrations of conjugated lectin respectively have stimulatory and inhibitory effects on C-fibre neurons. Figure 11 does require extrapolation along the x-axis. Nonetheless, the inventors of the present application confirm that such extrapolation along the x-axis in Figure 11 would result in inhibition of substance P release at a high lectin conjugate concentration. In other words, a high unconjugated lectin concentration (see Figure 10) and a high lectin conjugate concentration (see Figure 11) demonstrate inhibition of neurotransmitter or neuromodulator release of C-fibres.

Examples 5 and 6, as illustrated respectively by and Figures 2 and 3, are also consistent with such a conclusion. Figure 2 illustrates the analgesic effect of unconjugated lectin *in vivo*. Figure 3 confirms that a lectin conjugate also has an

analgesic effect *in vivo*, and that the presence of a companion peptide or protein does not adversely affect the efficacy of the lectin component.

Thus, so long as the requisite lectin is present, the above-described C-fibre inhibition and resulting therapeutic effects are achieved. In one embodiment, the lectin may take the form of a conjugate, wherein the lectin is coupled to a companion peptide or protein. However, since the lectin component is the active molecule, the presence of a companion peptide or protein makes no difference to the function of the lectin. In this regard, the companion peptide or protein acts as nothing more than an inert carrier or adjuvant.

The biological function and efficacy stems from the presence of lectin. No special consideration is necessary when preparing the conjugates. In this regard, conventional conjugation techniques would be adequate, and are well within the scope of a skilled person. By way of example, Applicants refer to their published international applications WO 94/21300, WO 96/33273, WO 99/17806 which detail suitable conjugation techniques. *See* Exhibit E. Particular reference is made to WO 99/17806, which describes the conjugation of a galactose-binding lectin to a companion peptide or protein.

D. Conclusion of Written Description Rejection

The Examiner is reminded that Applicants' claims are presumed to be supported by an adequate written description. Notwithstanding that a *prima facie* case for a written description rejection has not been set forth, the arguments presented by this Amendment and Reply have shown by a preponderance of the evidence that the skilled artisan would recognize that Applicants' written description adequately supports the claims.

Accordingly, Applicants request that the Examiner reconsider and withdraw the written description rejections.

II. Enablement Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 39-42 are rejected under 35 U.S.C. 112, first paragraph. In particular, the Examiner has alleged that

> . . . the specification, while being enabling (notwithstanding the straight Enablement Rejection) for a method of treating the C-fibre neuron associated diseases/conditions of pain and inflammation by an ECL conjugate (Figures 3 and 13), does not reasonably provide enablement for treatment of any C-fibre neuron associated diseases/conditions (claim 39), nor the distinctly claimed conditions of psoriasis and mucus hypersecretion (claim 42), by an ECL conjugate. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention commensurate in scope with these claims.

Office Action, page 5, first full paragraph. Hence, the Examiner's enablement rejection is directed to the *scope of use* of ECL conjugates. In particular, the rejection alleges that the specification does not enable the skilled artisan to use ECL conjugates to treat i) any C-fibre neuron associated diseases or conditions, ii) psoriasis or iii) mucus hypersecretion. Applicants respectfully traverse this rejection.

A. Requirement for Setting forth a Prima Facie Case of Non-Enablement

Reflecting the decisions of the federal courts, the M.P.E.P. provides guidance to examiners regarding enablement rejections. *See* M.P.E.P., 8th ed., § 2164 (Rev. 2, May 2004). In particular, the M.P.E.P. states that "[i]n order to make a rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention." *Id.* at §2164.04 (*citing In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993)). Moreover, any allegation doubting "the

truth or accuracy of any statement in a supporting disclosure . . . [must be supported by] acceptable evidence or reasoning which is inconsistent with the contested statement." *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367 (CCPA 1971). As with the written description requirement, an enablement analysis must be performed from the perspective of the skilled artisan: "The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. cir. 1988).

Moreover, the Federal Circuit has stated that "[t]he specification need not disclose what is well known in the art." *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991) (*see also Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986) ("a patent need not teach, and preferably omits, what is well known in the art."), *cert. denied*, 480 U.S. 947 (1987); *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984); *In re Myers*, 410 F.2d 420, 424 (C.C.P.A. 1969) ("A specification is directed to those skilled in the art and need not teach or point out in detail that which is well-known in the art.").

B. A Prima Facie Case for a Written Description Rejection Has Not Been Set Forth

The Examiner has not set forth a *prima facie* case that the claims are not enabled by the specification. The Examiner has not provided *any* evidence why the skilled artisan could only practice Applicants' invention with undue experimentation. Such evidence should begin with a description of the skilled artisan and his or her knowledge

to provide the proper perspective and framework from which to evaluate whether Applicants' claims are enabled. However, the Examiner entirely fails to define the skilled artisan or the knowledge generally known to him or her at the time the application was filed.

Apart from failing to define the skilled artisan, the Examiner has inaccurately assessed and analyzed the factors set forth in *In re Wands,* 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). For example, the Examiner alleges that

> A search of the prior art, as to ECL conjugates for treating C-fiber related disorders, revealed very limited number of teachings directed to the specific invention of the present application, therefore, the use of ECL conjugates for treating C-fiber related disorders cannot be construed as being well known in the art, and thus reliance for enablement must stem from the specification.

Office Action, page 6, lines 9-14. However, this analysis entirely misses the point. The relevant prior art is not ECL conjugates for treating C-fibre related disorders. *That is an aspect of Applicants' invention.* Rather, the relevant prior art pertains to diseases or conditions arising from C-fibre stimulation. As described in section ***I.B.3.*** above, there is no shortage of such teachings in Applicants' specification or the prior art. The art described in section ***I.B.3.*** also demonstrates that C-fibre stimulation is associated with psoriasis and mucus hypersecretion. Hence, the skilled artisan is aware that stimulation of C-fibres is associated with a panoply of diseases or conditions. The skilled artisan would accordingly understand that inhibition of such stimulation would mitigate or treat these diseases or conditions.

The Examiner's conclusions appear to rely solely upon alleged limitations of Applicants' exemplified teachings irrespective of the skilled artisan's familiarity with C-fibre neuron diseases or conditions:

> There are no working examples to indicate whether ECL conjugates would be enabled for treating the other [] 2 specifically claimed C-fibre neuron diseases/conditions (psoriasis and mucus hypersecretion) of claim 42 or any C-fibre neuron diseases/conditions as contemplated by claim 39.

Office Action, page 6, lines 17-20.

Applicant respectfully reminds the Examiner that courts have stated that working examples are not needed to enable the claims: "a specification need not contain a working example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without an undue amount of experimentation." *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (C.C.P.A. 1970) (citing *In re Long*, 368 F.2d 892 (C.C.P.A.1966)).

Moreover, the M.P.E.P. states that "if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate." M.P.E.P., 8th ed., § 2164.02 (Rev. 2, May 2004) (*citing In re Brana,* 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. cir. 1995)). Here, Applicants' working examples correlate to any diseases or conditions associated with the stimulation of C-fibre activity as well as psoriasis and mucus hypersecretion because i) the skilled artisan recognizes that the release of substance P constitutes C-fibre activity, and ii) the skilled artisan associates various diseases and conditions (including psoriasis and mucus hypersecretion) with C-fibre activity. Hence, because Applicants demonstrated that their lectin conjugates inhibit substance P, the skilled artisan would accept that Applicants' claims are enabled.

With respect to the claim breadth and quantity of experimentation needed, the Examiner has summarily (and unfairly) concluded that

> Absent sufficient teachings in the specification or art sufficient to overcome the teachings of unpredictability in the art as to enablement on whether any C-fibre neuron associated diseases/conditions may be treated using an ECL conjugate; it would require undue experimentation by one of skill in the art to be able to practice the invention commensurate in scope with the claims.

Office Action, page 7, lines 3-7.

Applicants request that the Examiner identify those "teachings of unpredictability in the art" as none have been provide in any Office Communication. It is unclear to Applicants exactly what the Examiner is alleging to be unpredictable. There is nothing unusual about the formulations or modes of administration of the present invention, which would therefore also be considered conventional to a skilled person. Moreover, as extensively described in section ***I.B.3.***, Applicants' specification and the prior art make it abundantly clear that there is nothing unpredictable about correlating C-fibre activity with diseases or conditions. Finally, there is nothing unpredictable about the premise that inhibition of a biochemical or biological activity associated with a disease or condition constitutes treatment or mitigation of that disease. Thus, the Examiner's conclusion regarding undue experimentation is unfounded and baseless.

3. The Specification Enables the Claims for the Skilled Artisan

Notwithstanding that a *prima facie* case for a written description rejection has not been set forth, Applicants reiterate the following statements regarding the skilled artisan for the convenience of the examiner. Prior to the present application, the link between undesirable stimulation of C-fibre neuron activity (i.e., undesirable release of neurotransmitters or neuromodulators, such as substance P) and corresponding medical diseases or conditions was known. *See* ***I.B.3.*** Applicants' specification identifies new

and nonobvious agents capable of inhibiting such stimulation, and as such, have enabled the skilled artisan to treat C-fibre diseases or conditions without undue experimentation. Applicants' specification is presumed to be enabled. Moreover, this Amendment and Reply has demonstrated by a preponderance of the evidence that the claims are enabled. Accordingly, Applicants request that the Examiner reconsider and withdraw the enablement rejection.

III. Indefiniteness Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 39-42 are rejected under 35 U.S.C. 112, second paragraph. In particular, the Examiner alleges that these claims are "indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Office Action, page 7, lines 13-14.

Solely to expedite prosecution and not in acquiescence to the rejections, Applicants have cancelled claims 39-42 and presented claims 48-57. Applicants request that the Examiner reconsider and withdraw this rejection, which is now moot.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will

expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Neurobiology of Nociceptors

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14 Functional properties of human cutaneous nociceptors and their role in pain and hyperalgesia

H.E. TOREBJÖRK, M. SCHMELZ, AND H.O. HANDWERKER

Introduction

The study of peripheral mechanisms of pain was greatly advanced by the technique of microneurography introduced by Hagbarth and Vallbo (1967). This technique not only makes possible experiments on human volunteers instead of on guinea-pigs, but also adds a psychophysical dimension to electrophysiology, since human volunteers are able to describe their stimulus-induced sensations while primary afferent units are recorded. From this additional information one may deduce whether the excitation of a particular type of nerve fibre can possibly contribute to the respective sensory experience. For instance, combined psychophysical and neurophysiological experiments in humans indicate that C-fibre mechanoheat (CMH) nociceptors provide a peripheral neuronal basis for the determination of the heat pain threshold, at least in hairy skin (Van Hees and Gybels 1981; LaMotte *et al.* 1982). Furthermore, a nearly linear relation found between the mean suprathreshold response functions in a population of CMH nociceptors and the median ratings of pain suggests that these nociceptors provide an essential code for the magnitude of pain sensation in response to heat (Torebjörk *et al.* 1984*a*). This is supported by the finding that CMH nociceptors are activated by small increments of 0.1–0.5°C on a base temperature of 48°C, which is matched by the human ability to detect such increments as painful (Robinson *et al.* 1983).

A typical feature of CMH nociceptors is their sensitization after heat injury to their receptive fields. The lowering of thresholds to heat stimuli and the enhanced responses to suprathreshold stimuli correlate with increased pain ratings, indicating that CMH nociceptors contribute to heat hyperalgesia in hairy skin (LaMotte *et al.* 1982; Torebjörk *et al.* 1984*a*). The situation seems to be different in the glabrous skin of the hand, since neurophysiological studies in monkey have shown that only sensitization of Aδ nociceptors matches the heat hyperalgesia following a burn lesion, while CMH nociceptors are often desensitized (Meyer and Campbell 1981).

The microneurographic recording technique has been supplemented by electrical intraneural microstimulation of single (or small groups of) afferent fibres that were characterized by recording beforehand (Torebjörk and Ochoa 1980; Vallbo 1981). Microstimulation of nociceptor units provided insights into the quality of sensations induced by stimulation of certain types of afferent nerve fibres and into the role of their impulse patterns for sensation. Thus, intraneural microstimulation in C-fibre recording sites evoked dull pain sensations (Ochoa and Torebjörk 1989) that were projected with an accuracy of the order of 1 or 2 cm to the innervation territory of the recorded C

15 Neural mechanisms of primary hyperalgesia

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Hyperalgesia is a consistent characteristic of tissue injury and inflammation. For example, pain in response to gentle warming and light touch is common after a sun burn. Pharyngitis is associated with hyperalgesia in the pharyngeal tissues, such that merely swallowing induces pain. In inflammatory arthritis, slight movement of the joint leads to pain. Micturition in the presence of a urinary tract infection is painful, again reflecting the presence of hyperalgesia. In this chapter, we will focus our attention on the hyperalgesia that develops in cutaneous tissue. We will discuss how hyperalgesia depends on: (1) the stimulus modality being tested (for example, heat, cold, mechanical, or chemical stimuli); (2) the type of injury; (3) the type of cutaneous tissue (for example, hairy versus glabrous skin); and (4) neurogenic factors (for example, axonal reflex, sympathetic efferents).

Hyperalgesia occurs not only at the site of injury but also in the surrounding uninjured area. Hyperalgesia at the site of injury is termed *primary hyperalgesia*, while hyperalgesia in the uninjured skin surrounding the injury is termed *secondary hyperalgesia* (Lewis 1942). In this chapter, we will discuss the evidence that primary hyperalgesia is due, in large part, to the sensitization of primary afferent receptors. As will be discussed in detail later (LaMotte, Chapter 16, this volume), secondary hyperalgesia appears to be due to sensitization in the central nervous system.

Taxonomy: hyperalgesia versus allodynia

One of the first issues to address is nomenclature. The IASP Subcommittee on Taxonomy has recommended that hyperalgesia be defined as an increased response to a stimulus that is normally painful and that hyperalgesia should not be associated with a lowering of threshold (Merskey and Bogduk 1994, pp. 210–12). It is suggested that 'allodynia' refer to pain due to a stimulus that does not normally produce pain.

The term 'allodynia' stems from the Greek term *allos*, which means 'other' or 'another' and is a common prefix for medical conditions that are different from the expected. The term allodynia appears to be appropriate when pain arises from channels of perceptual information not ordinarily concerned with pain. For example, a patient may be said to have allodynia if pain arises from activity in low-threshold mechanoreceptors.

The distinction between hyperalgesia and allodynia suggested by Merskey and Bogduk (1994) poses many difficulties. The term hyperalgesia has been in the literature for decades and was used by Lewis (1942) and Hardy and co-workers (1952) to refer to a lowering of the pain threshold as well as increased pain in response to suprathreshold stimuli. If secondary hyperalgesia develops because low-threshold receptors attain the capacity to evoke pain, should the term secondary hyperalgesia be discarded and replaced by the term secondary allodynia?

16 Secondary cutaneous dysaesthesiae

ROBERT H. LAMOTTE

A localized irritation of the skin, if sufficiently potent, produces a remarkable change in the quality of mechanically evoked sensations in a large area surrounding the injury. In the case of a particularly noxious stimulus—a burn, a cut, or crush—the skin becomes hyperalgesic: normally painful stimuli such as a pinprick elicit abnormally intense and prolonged sensations of pain (punctate hyperalgesia), and the threshold for pain may be lowered so that normally innocuous stimuli such as a light touch are painful ('allodynia') (Lewis 1936; Hardy *et al.* 1950; LaMotte *et al.* 1991; Koltzenburg *et al.* 1992; Meyer *et al.* 1993).

In the case of an itch-producing irritation such as a mosquito bite, the quality of mechanically evoked sensations in the skin surrounding the region of wheal and local redness is also changed. But instead of hyperalgesia, a state of hyperknesis is produced ('knesis' from the ancient Greek word for itching). A stimulus that can evoke itch in uninjured skin (such as a prickly hair) now elicits an abnormally intense itch (punctate hyperknesis) (unpublished observations) and gently stroking the skin with an innocuous cotton swab evokes itch and/or exacerbates an ongoing itch ('alloknesis') (Bickford 1938; Graham *et al.* 1951; Simone *et al.* 1987, 1991*a*; LaMotte 1992).

The unpleasant, abnormal sensory states (dysaesthesiae) of hyperalgesia and hyperknesis are qualitatively very different as illustrated by the different sensations and reactions in response to the same stimuli. In hyperalgesic skin, a prickly hair elicits pain as does light stroking with a cotton swab. These unpleasant sensations are accompanied by behavioural reactions of protecting the skin from further mechanical contact. In contrast, when the same stimuli are applied to hyperknesic skin, they evoke sensations of itch and reactions of scratching the stimulated area. The dysaesthesiae of hyperalgesia or hyperknesis when elicited within the area of cutaneous irritation are called 'primary'. Those evoked by stimuli delivered outside this primary area are termed 'secondary' (Hardy *et al.* 1950, 1951). In the following, I will discuss the contributions of primary cutaneous peripheral nerve fibres to the cutaneous dysaesthesiae of secondary hyperalgesia and secondary hyperknesis.

Sensory descriptions of experimentally produced cutaneous secondary dysaesthesiae

Secondary hyperalgesia

Cutaneous secondary hyperalgesia can easily be produced in the laboratory by a variety of localized injuries of the skin including those resulting from electrical, thermal, mechanical, and chemical stimuli (Lewis 1936; Hardy *et al.* 1950). It can also be induced by the electrical stimulation of a cutaneous nerve (Lewis 1936), although it is then uncertain to what extent the hyperalgesia arises from resulting injury of tissues in

17 Efferent function of nociceptors

BRUCE LYNN

Introduction

Nociceptors are defined as afferent fibres signalling strong, injury-threatening stimuli or the presence of chemical irritants, including many inflammatory mediators. Yet, uniquely amongst afferent neurones, many nociceptors also have efferent actions in the tissue that they innervate. When excited, they release vasoactive peptides with potent actions on local blood vessels and on cells of the immune system. This phenomenon is termed neurogenic inflammation. Additionally, in some tissues substances released from nociceptive terminals may cause smooth muscle contraction, and in the airways may trigger the secretion of mucus. In this chapter several aspects of these efferent actions of nociceptors will be examined. First, a brief historical review will introduce the main features of neurogenic inflammation. Next, the question of which nociceptors are involved in different efferent actions will be addressed. The way these neurones can be selectively excited and often functionally blocked by capsaicin will be examined. Then, the nature of the released substances and their actions will be considered. Finally, the evidence for a significant role for nociceptors (1) in triggering inflammation and (2) in the pathophysiology of some diseases (for example, arthritis, asthma) will be reviewed.

Historical background

That some somatosensory neurones had vasodilator actions was known before there was any clear definition of afferent classes such as nociceptors. Vasodilatation in response to stimulation of the dorsal (sensory) roots was first reported by Stricker in 1876 and was studied in detail by Bayliss (1901, 1902) who described clearly the main features (Fig. 17.1). This phenomenon of antidromic vasodilatation was shown to involve fibres with cell bodies in the posterior roots, to occur with relatively low-frequency stimulation, and to be slow in onset and recovery. Interest at this time centred on how this observation contradicted the Bell–Magendie law of separation of function (that is, dorsal roots sensory; ventral roots motor) and on the possible role of such fibres in reflex control of blood flow. Bayliss in fact presents indirect evidence for a role in reflex vasodilatation following stimulation of aortic baroreceptors (Bayliss 1902). Direct evidence for central activation of dorsal root afferents has, however, not been forthcoming. A clear description of how such fibres might be activated was provided by Bruce (1913) (Fig. 17.2). Bruce was studying the local inflammatory reaction following application of the chemical irritant mustard oil and found that this was reduced or abolished by chronic sensory denervation or by application of local anaesthetics. He therefore proposed that some afferent fibres might have, in addition to their afferent terminals, a second type of terminal that was motor to blood vessels (Fig. 17.2). The terminals on blood vessels would be activated following stimulation of the sensory

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18 Nociceptors and neuroimmune interactions

CHRISTOPH STEIN

Introduction

Neuroimmune interactions have been described extensively in the central nervous system. Commonly, such interactions refer to the effects of compounds released from immune-competent cells upon central neurones or vice versa. The concept of an interrelation between peripheral sensory nerves and immune cells is relatively novel. This has been proposed by a number of authors, based mostly on microanatomical findings in inflammatory processes (reviewed in Weihe *et al.* 1990). Similarly to the situation in the central nervous system, mutual interactions may take place, that is, substances released from peripheral nerves may act on immune cells or vice versa. Effects resulting therefrom include immunomodulation and pro- or antiinflammatory, hyperalgesic or analgesic phenomena. This chapter is divided into two main parts, the first part covering substances released from peripheral neurones and the second part covering substances derived from immune cells. The focus will be on functional studies examining the crosstalk between cells of the immune system and peripheral sensory nerves. Auto- or paracrine actions of the respective compounds will not be discussed in detail.

Neurone-derived substances

Substance P

The most extensively studied compound is substance P (SP). SP is contained in dorsal root ganglion (DRG) neurones and is released from their peripheral and central terminals (see Lawson, Chapter 3, this volume). Receptors for SP have been demonstrated on T and B lymphocytes by cell-sorter and radioligand-binding techniques (McGillis *et al.* 1987, 1990; Bost and Pascual 1992). Moreover, messenger ribonucleic acid (mRNA) encoding SP receptor is constitutively expressed in mast cell lines (Ansel *et al.* 1993), in lymphocytes (Bost and Pascual 1992), and in macrophages (Bost *et al.* 1992). Apparently, the SP receptors expressed by immune cells are very similar to those expressed by neurones, as evidenced by radioreceptor binding assays and determination of the gene encoding the SP receptor (Bost and Pascual 1992; Bost *et al.* 1992).

So far, the majority of studies indicate that the effects of SP on cell-mediated immunity are stimulatory. SP has been shown to modulate immediate hypersensitivity responses by stimulating the generation of arachidonic acid-derived mediators from mast cells (McGillis *et al.* 1987). Furthermore, SP may influence mast-cell-mediated late inflammatory events by modulating the production and secretion of several cytokines. Thus, SP can induce mRNA for tumour necrosis factor α (TNFα) and stimulate TNF secretion in a murine mast-cell line or in freshly isolated mast cells (Ansel *et al.* 1993). Chemotaxis, lysosomal enzyme release, and phagocytosis by mononuclear and polymorphonuclear leucocytes are altered by SP (McGillis *et al.* 1987). SP enhances the synthesis of DNA,

Textbook of Pain

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EXCITATORY AMINO ACIDS

Glutamate receptors are expressed in dorsal root ganglion cells (Sato et al 1993b) and have also been identified on peripheral terminals of cutaneous nociceptors (Carlton et al 1995). The peripheral application of glutamate activates nociceptors (Ault & Hildebrand 1993) and the peripheral administration of ligands binding to glutamate receptors induces pain behaviours in animals (Zhou et al 1996). An involvement of peripheral glutamate receptors in formalin-induced pain behaviours has been demonstrated (Davidson et al 1997). Intra-articular injection of excitatory amino acids and inflammatory agents results in signs of hyperalgesia that are reversed by glutamate receptor antagonists (Lawand et al 1997). However, the peripheral algogenic action of glutamate is unclear, as it failed to excite nociceptors and it does not induce pain when injected into human skin (Vyklický & Knotková-Urbancová 1996).

OPIOIDS

Besides its central analgesic action, morphine and other opioids produce analgesia in inflamed tissues by a peripheral mechanism (Stein et al 1988, 1989). Opioid receptors have been demonstrated on peripheral terminals of afferent fibres (Stein et al 1990, Coggeshall et al 1997) and axonal transport of these receptors is enhanced during inflammation (Hassan et al 1993). Peripheral analgesia by opioids appears to be part of a physiological antinociceptive system, because increased amounts of endogenous opioids have been found in inflamed tissues (Stein et al 1990). Inflammatory cells such as macrophages, monocytes and lymphocytes contain opioid peptides (Przewlocki et al 1992). The release of endogenous opioids and antinociception can be induced by interleukin 1β and corticotropin-releasing hormone originating from the inflamed tissue (Schäfer et al 1994, 1996).

EFFERENT FUNCTIONS OF NOCICEPTORS

Nociceptors, apart from signalling pain, serve regulatory and trophic functions (Kruger 1988, McMahon & Koltzenburg 1990). This might explain the finding that small-diameter fibres outnumber large-diameter fibres by a factor of 4 (Ochoa & Mair 1969). An efferent role for nociceptors was suggested by several investigators almost a century ago (for a historical review see Lynn 1996; Lewis 1937). Two efferent cutaneous phenomena have been identified that depend on the integrity of afferent nociceptive fibres and are part of the so-called neurogenic inflammation (Jansco et al 1967, 1968): vasodilatation, which becomes visible as a flare surrounding a site of injury, and plasma extravasation, which may become apparent as a wheal at the site of injury. Both phenomena are mediated by vasoactive neuropeptides (SP, CGRP) that are released from peripheral terminals of nociceptors upon activation. SP and CGRP also play a role in immunological processes (e.g., the migration of leucocytes at sites of tissue injury (Nilsson et al 1985, Kjartansson et al 1987), and they stimulate the epidermal cells (e.g., keratinocytes and Langerhans cells) which are necessary for the maintenance and repair of skin integrity (Hsieh et al 1996, Kruger 1996). Afferent fibres are also considered to play a role in the regulation of activity of autonomic ganglia and visceral smooth muscles (for reviews see Maggi & Meli 1988, Lynn 1996, Szolcsanyi 1996, Holzer 1998). Afferent fibres therefore serve a trophic efferent function in somatic and visceral tissues (for review see Kruger 1996).

The principal lines of evidence indicating that afferent neurons are involved in neurogenic inflammation are:

1. The responses are abolished by surgical or chemical ablation (e.g., capsaicin) of the sensory innervation of the involved tissues (Jansco et al 1977, Lembeck & Holzer 1979, Gamse et al 1980, Carpenter & Lynn 1981, Pinter & Szolcsanyi 1995).
2. The responses occur independent of the autonomic nervous system (Couture et al 1985, Blumberg & Wallin 1987).

The fibres involved in the reflex vasodilatation are polymodal nociceptive C fibres that are capsaicin sensitive (Jansco et al 1967, 1968). Stimulation of Aδ fibres may also result in a flare response (Jänig & Lisney 1989, Kolston & Lisney 1993).

Flare is thought to be caused by a peripheral axon reflex. The activation of one branch of a nociceptor by a noxious stimulus results in the antidromic invasion of action potentials into adjacent branches of the nociceptor which, in turn, causes the release of vasoactive substances from the terminals of the nociceptor. However, the extent of the flare far exceeds the size of the receptive fields of conventional nociceptors (Beitel & Dubner 1976, Kumazawa & Perl 1977a,b, Campbell & Meyer 1983, Raja et al 1984, Treede et al 1990b). Possible explanations for this discrepancy might include:

1. Flare is mediated by a subpopulation of chemosensitive nociceptive fibres with large receptive fields (Lewis 1937). Some C fibres with large, complex receptive fields have been reported (Meyer et al 1991, Schmelz et al 1997).

2. Flare results from spreading depolarization along adjacent nociceptive terminals via a daisy-chain cascade mechanism (Lembeck & Gamse 1982).
3. Axo-axonal coupling between small fibres may be responsible for the spread of the flare reaction (Mathews 1976, Meyer et al 1985b).

Several lines of evidence indicate that the neural substrates for vasodilatation and the perception of pain are different.

1. The magnitude of vasodilatation induced by a noxious stimulus does not always increase with the intensity of pain (Koltzenburg & Handwerker 1994).
2. Low activity (<1 Hz) in C fibres can generate significant vasodilatation (Lynn & Shakhanbeh 1988) which, in man, does not cause any conscious sensation (Gybels et al 1979).
3. Histamine can produce a large flare with little or no pain (Treede 1992).

Possible explanations include: (i) different discharge patterns are needed for pain versus flare in a given fibre population or (ii) certain classes of afferents are better designed for flare than pain and vice versa.

Recent evidence suggests that the antidromic activity involved in the effector responses can originate from the spinal cord. A series of studies in a model of acute arthritis indicates that primary afferent input to the spinal cord activates multisynaptic central neuronal pathways that in turn influence the development of neurogenic inflammation (Sluka and Westlund 1993, Sluka et al 1994a,b, for review see Sluka et al 1995b). The activation of primary afferent fibres may result in depolarization of the central terminals of other afferent fibres (PAD). If the PAD is large enough (e.g., under peripheral inflammatory conditions), the depolarization can be sufficient to initiate action potentials at the central terminals that are conducted antidromically in the primary afferent fibres (dorsal root reflexes, DRRs). It is postulated that the antidromic impulses (DRRs) triggered by PAD result in release of neuropeptides in the joint from peripheral terminals of the afferents and contribute to the inflammatory process. DRRs have been recorded in C-, Aδ- and Aβ-fibre types in rat models of acute arthritis (Rees et al 1995, Sluka et al 1995a). The joint inflammation and the DRRs were attenuated by prior dorsal rhizotomy (Sluka et al 1994b, Rees et al 1995). The increase in blood flow that has been measured in response to painful stroking stimuli applied to the zone of secondary hyperalgesia appears to be a human correlate of the dorsal root reflex (Cervero & Laird 1996a).

Anatomical, immunological and histochemical studies have revealed the presence of several peptides in sensory neurons and their peripheral and central projections. These peptides include substance P and other tachykinins such as neurokinins A and K, CGRP, somatostatin and vasoactive intestinal polypeptide (Lembeck & Gamse 1982, Holzer 1988, Micevych & Kruger 1992). The presence and release of neuropeptides from capsaicin-sensitive sensory nerve endings, their ability to induce many of the signs of acute inflammation, including vasodilatation and plasma extravasation, and the inhibition of neurogenic vasodilatation by selective neuropeptide antagonists indicate that they are the principal mediators of neurogenic inflammation and axon reflexive flare (Saria 1984, Helme et al 1986, Lembeck & Donnerer 1992, Escott & Brain 1993). The vasodilatation induced by substance P may, at least in part, be an indirect effect related to histamine release from mast cells (Hagermark et al 1978, Barnes et al 1986, Ebertz et al 1987). CGRP also has potent and prolonged vasodilator properties in humans and may be one of the mediators of neurogenic vasodilatation, possibly playing a role in the long-term vascular responses to injury (Brain et al 1985, 1986, Piotrowski & Foreman 1986, Pedersen-Bjergaard et al 1991. Other efferent actions of nociceptors that are mimicked by vasoactive neuropeptides are contraction of smooth muscles, stimulation of mucous secretion from airways and leucocyte adhesion (Lundberg 1993, Smith et al 1993, Ramnarine et al 1994). Some efferent functions of nociceptors and the chemical mediators involved in cutaneous and visceral tissues are shown in Figure 1.20.

Substance P, neurokinin A and CGRP are also released from trigeminovascular axons in the pial and dural circulations, resulting in vasodilatation and plasma extravasation (Moskowitz et al 1983, 1989). This release of vasoactive neuropeptides from perivascular sensory nerves via axon reflex-like mechanisms may play an important role in the pathophysiology of vascular headache and cerebral hyperperfusion syndromes (MacFarlane et al 1991, Moskowitz 1991). Other diseases in which a neurogenic component is suspected include rheumatoid arthritis, asthma, inflammatory diseases of the gastrointestinal tract and ocular inflammatory disease (see Maggi et al 1993).

NEURAL MECHANISMS OF ITCH

Itch is the common sensory phenomenon associated with the desire to scratch. Like pain, itch can be produced by chemical, mechanical, thermal or electrical stimuli. However, itch differs from pain in that itch can be evoked only from the superficial layers of skin, mucosa and

1: J Invest Dermatol. 1989 Jan;92(1):126-9.

Related Articles, Links

Comment in:

- J Invest Dermatol. 1989 Nov;93(5):708.

Quantification of cutaneous sensory nerves and their substance P content in psoriasis.

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The aim of the present study was to extend our previous hypothesis that the inflammatory reaction in psoriasis is neurogenic, and that substance P mediates the inflammation. For this purpose, the pattern of neurofilament-positive sensory nerve fibers was studied and the lengths and substance P content of these fibers measured morphometrically in dermal and epidermal compartments of the psoriatic lesion, psoriatic but lesion-free skin, and control skin. The epidermis and dermis of the psoriatic lesions were significantly more densely innervated with neurofilament-positive fibers than either lesion-free psoriatic or control skin. Although substance P is known to be rapidly degraded in tissues, and its actual concentrations in the sections were unknown, there was an increase in substance P containing nerves in the psoriatic lesion, the increase being significant in the epidermal nerve fibers. No significant differences in the measured parameters were obtained between lesion-free psoriatic and control skin. These results indicate that there is an altered pattern of sensory nerves in a psoriatic plaque and that substance P may be an important mediator in the inflammatory processes that contribute either to the initiation or maintenance of a psoriatic lesion.

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1: J Am Acad Dermatol. 1986 Feb;14(2 Pt 1):305-11.

Stress, symmetry, and psoriasis: possible role of neuropeptides.

Farber EM, Nickoloff BJ, Recht B, Fraki JE.

The role of stress as a triggering factor in the exacerbation of psoriasis and the clinically symmetric distribution of psoriatic plaques suggested a possible role for neuropeptides in the etiopathogenesis of psoriasis. Several observations by other investigators involving substance P suggested to us a possible role for substance P as a modulator of the inflammatory response in psoriasis. A hypothesis for the role of substance P that would account for the temporal onset with stress, the clinical symmetry of lesions, and the histopathologic features of psoriasis is presented.

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(54) Title: NOVEL AGENT FOR CONTROLLING CELL ACTIVITY

(57) Abstract

This invention describes a novel agent for the targeted control of a mammalian cell activity, in particular the agent is used to control the interaction of particular cell types with their external environment. The agent has applications as a pharmaceutical for the treatment of a variety of disorders. An agent according to the invention comprises three Domains B, T and E linked together in the following manner: Domain B--Domain T--Domain E where Domain B is the Binding Domain which binds the agent to a Binding Site on the cell which undergoes endocytosis to produce an endosome, Domain T is the Translocation Domain which translocates the agent (with or without the Binding Site) from within the endosome across the endosomal membrane into the cytosol of the cell, Domain E is the Effector Domain which inhibits the ability of the Recyclable Membrane Vesicles to transport the Integral Membrane Proteins to the surface of the cell.

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NOVEL AGENT FOR CONTROLLING CELL ACTIVITY

TECHNICAL FIELD

This invention describes a novel agent for the targeted control of mammalian cell activity, in particular the agent is used to control the interaction of particular cell types with their external environment. The agent has applications as a pharmaceutical for the treatment of a variety of disorders.

BACKGROUND

A fundamental property of living cells is their ability to respond to their external environment. The interface between a cell and its external environment is the plasma membrane. The plasma membrane consists of a phospholipid bilayer in which many kinds of protein molecules are embedded. These integral membrane proteins (IMPs) are responsible for many of the interactions of a cell with its external environment.

The interactions in which the IMPs are involved include: the transport of materials, including nutrients, into and out of the cell; the regulated permeability of the plasma membrane to ions; the recognition of, and response to, extracellular molecules; and the adhesion of one cell to another cell. A specialised function of the immune system, that is also mediated via IMPs, is the display of particular foreign peptide sequences by one group of immune cells to another group.

One of the ways in which a cell regulates its ability to respond to, and interact with, the external environment is by changing the quantity and types of IMPs present at the plasma membrane. One mechanism by which this is achieved is the reversible internalisation of IMPs via an endocytotic pathway into Recyclable Membrane Vesicles (RMVs). In these cases IMPs stored in the RMVs represent an internal store or pool of IMPs available for rapid export to the cell surface via a process of exocytotic fusion of the RMVs with the plasma membrane. Modulation of the equilibrium of this exocytotic / endocytotic

cycle allows rapid regulation of the density of IMPs present at the cell surface. In one example of the process for controlling cell activity, the uptake of glucose by insulin-responsive cells in skeletal muscle and adipose tissue is regulated. Insulin increases the amount of a particular isoform of glucose transporter, GLUT4, which is found in the plasma membrane of these cells. The higher concentration of GLUT4 molecules at the surface of the cell results in increased uptake of glucose. Therefore, by controlling the number of glucose transporters present in the plasma membrane the response to insulin can be modulated.

Another example of alterations in cell surface IMP expression in response to external signals is that of the receptor for the complement fragments C3b and C4b, the type 1 complement receptor CR1. Upon activation of human neutrophils the plasma membrane expression of CR1 is transiently increased 6- to 10- fold.

In another example a number of inflammatory and immune cells modify their expression of cell surface adhesion molecules upon activation. Hence, activation of neutrophils or monocytes leads to a modulation of the cell surface adhesion molecules Mac-1 and p150,95. These adhesion molecules are important in the targeting and movement of inflammatory cells to sites of inflammation.

In yet another example, a variety of hormones (insulin, insulin-like growth factor, interleukin 1 and platelet-derived growth factor) cause a rapid increase in the cell surface expression of the transferrin receptor in a variety of cell types. The transferrin-receptor binds diferric transferrin from the external environment of the cell, and is thereby involved in the uptake of iron by cells. This transferrin/transferrin-receptor system may also play a role in the transcellular movement of iron into the CNS across the blood brain barrier, a process known as transcytosis. Transcytosis is also involved in the transfer of maternal immunity to the developing foetus.

In yet another example the diuretic hormone aldosterone is known

to increase the cell surface expression of Na^+ channels in the apical membrane of urinary bladder epithelial cells. This mechanism is involved in salt retention and occurs, for example, in conditions of low sodium-containing diets.

In a further example of the modulation of cell membrane expression of IMPs, it is noted that the function of the immune system is based upon the recognition of foreign, or non-self, antigens. Part of this recognition and immune response is provided by cells of the immune system able to recognise and respond to foreign peptide sequences. These peptide sequences are presented to the immune cells by other cells of the immune system known as antigen presenting cells. Antigen presenting cells ingest foreign antigenic proteins, digest these to peptides and display the foreign peptides in a cleft formed at the cell membrane by IMPs of the major histocompatibility complex.

Thus IMPs are central to a cell's ability to interact with its external environment and, given the diverse and varied nature of these interactions, it is not surprising to discover that there are a vast array of different IMPs. The pivotal role of IMPs in a cell's function means that they are often involved in pathophysiologic states, and are the target for many therapeutic interventions.

Prior art approaches to the control of IMP activity have mainly focused on modulating the function of the IMP once expressed at the cell surface. Thus prior art therapeutic interventions tend to be specific for particular IMPs and for particular functions of particular cell types. Inhibitors of specific transport IMPs have been developed as therapeutic agents. For example, inhibitors of the 5HT transport protein of neurones are used as anti-depressants. Antagonists of particular receptor IMPs are very commonly used pharmaceutical agents. Examples include antihistamines, both those specific for the H1 and the H2 subtypes of histamine receptor, and antagonists of the ß-adrenoceptor. Inhibitors of IMP function are also widely used as pharmaceutical agents. Examples include inhibitors of

transmembrane ion movements such as the diuretics furosemide and amiloride, the latter of which is an inhibitor of the bladder epithelial cell apical Na^+ channel. Inhibitors of potassium channels are known to be under development as antiarrhythmic agents. Cell adhesion IMPs are also currently targets for the development of selective antagonists.

Another approach being pursued is to selectively modify the expression of particular IMPs at the genetic level by alteration of the level of transcription of the appropriate gene coding for that IMP and hence modulation of specific IMP protein synthesis.

In summary, IMPs are known to play a critical role in the response of a cell to its external environment. Previous approaches to the control of IMPs have generally involved the targeting of a specific IMP at the cell surface and modifying its functional capacity. The control of the density of IMPs within the plasma membrane is anticipated to have broad applications in the treatment of a variety of disorders. In view of the great diversity of IMPs and the particular nature of current therapeutic interactions it is the surprising discovery of the current invention that a single class of agents can modify the expression of IMPs in a wide variety of cell types. The same class of agent is also able to modify the expression of transport IMPs, receptor IMPs, adhesion IMPs, channel IMPs and antigen presenting IMPs. Previously, agents affecting IMPs have been classified by function, for example Ca^{++} antagonists, the members of each group being chemically and mechanistically very diverse. The class of agent referred to in the current invention, by contrast, is structurally homogeneous, with rationally introduced substitutes of particular domains having predictable effects on the function of the agent. A further aspect of the invention is that the agent can be selectively targeted to particular types of cell to allow selective modulation of IMP expression only in that cell type.

STATEMENT OF INVENTION

The current invention relates to an agent for controlling the

interaction of a cell with its external environment. Specifically, the invention provides an agent for controlling the transport of Integral Membrane Protein (IMP) molecules from the internal components of a cell to the cell membrane, so as to modify the cell's interaction with its external environment. More specifically the invention provides a novel agent which modifies the structure of Recyclable Membrane Vesicles (RMVs) such that their ability to transport IMPs to the surface of the cell is inhibited.

Definitions

The following terms have the following meanings;

Integral Membrane Protein (IMP) means any protein which is embedded in and spans across the lipid bilayer of a biological membrane

Recyclable Membrane Vesicle (RMV) means an intracellular vesicle, present in the cytosol of a cell, bounded by a lipid bilayer membrane . RMVs are formed from the plasma membrane and move into the cell interior by a process referred to as endocytosis. RMVs undergo a cyclical process of forming from and fusing with the cell plasma membrane. The process of moving to and fusing with the plasma membrane is referred to as exocytosis. The function of RMVs in the cell is in the reversible transport of IMPs to and from the cell surface; in this they are distinct from the secretory vesicles of neurosecretory cells.

Endosome means those intracellular vesicles which have formed from the plasma membrane by a process of endocytosis.

Heavy chain means the larger of the two polypeptide chains which form Clostridial neurotoxins; it has a molecular mass of approximately 100 kDa and is commonly referred to as HC. Light chain means the

smaller of the two polypeptide chains which form Clostridial neurotoxins; it has a molecular mass of approximately 50 kDa and is commonly referred to as LC. Naturally occurring Heavy and Light chains are covalently coupled via at least one disulphide bond.

H_2 fragment means a fragment derived from the amino terminal end of the Heavy chain of a Clostridial neurotoxin by proteolytic cleavage for example with trypsin or papain.

H_2L means a fragment of a Clostridial neurotoxin produced by proteolytic cleavage for example with trypsin or papain in which the Light chain is still coupled via disulphide bonds to the H_2 fragment.

In one aspect of the invention an agent is provided for the control of the level of the IMP responsible for the transport of a metabolite across the cell membrane, so controlling the availability of that metabolite within the cell.

In another aspect of the invention an agent is provided for the control of the level of the IMP responsible for the transport of a metabolite across the cell membrane into the cell and out of the cell, so controlling the transport of that metabolite through the cell.

In yet another aspect of the invention an agent is provided for the control of the level of the IMP responsible for the selective permeability of the plasma membrane of the cell to an ion, so modulating the concentration of that ion within the cell.

In yet another aspect of the invention an agent is provided for the control of the level of the IMP responsible for the recognition of a signalling molecule, so modulating the responsiveness of the cell to that signalling molecule.

In yet another aspect of the invention an agent is provided for

the control of the level of the IMP responsible for the transduction of signals across the cell membrane following binding to the membrane of a signalling molecule, so modulating the responsiveness of the cell to that signalling molecule.

In yet another aspect of the invention an agent is provided for the control of the level of the IMP responsible for the display on the cell surface of peptide fragments derived from ingested antigens. The result of this in an organism is to affect the immune response of that organism.

The invention also provides an agent which has target specificity for target cell types so that the scope of the effect of the agent is limited to said cell types.

As previously stated, prior art approaches to the control of IMP activity have mainly focused on modulating the function of the IMP once expressed at the cell surface. In direct contrast the present invention modulates the level of IMP which becomes expressed at the cell surface.

DETAILED DESCRIPTION OF THE INVENTION

It can be seen that the object of this invention, to provide an agent for controlling the level of IMPs at a cell surface, has many potential applications for modulating the response of a cell to its environment. This invention includes an agent which functions so as to affect the mechanisms by which IMPs are carried to the surface of a cell, as evidenced in the examples, e.g. example 1 and 2. Such an agent must accomplish three discrete functions, the first two of which are known in the art. Firstly it must bind to a cell surface structure (the Binding Site). It must then enter into the cytosol of the cell. The entry of molecules into the cell is known to occur by a process of endocytosis. However, as only certain cell surface Binding Sites are known to be involved in endocytosis, only these Binding Sites are suitable as targets. Once taken into the cell by endocytosis the agent must then leave the resulting endosome across the endosomal membrane to enter the cytosol. The ability to achieve specific cell binding and entry of agents into the

cytosol is well known in the literature (for example: Pastan, I; Willingham, MC; & Fitzgerald, DSP, 1986, Cell 47, 641 - 648, Olsnes, S; Sandvig, K; Petersen, OW; & Van Dews, B, 1989, Immunol. Today 10, 291 - 295, Strom, TB; Anderson, PL; Rubin-Kelley, VE; Williams, DP; Kiyokawa, T; & Murphy, JR; 1991, Ann NY Acad. Sci 636, 233 -250). The third function of the agent is the surprising finding of this invention, namely the ability to affect the RMV. The further surprising aspect of this agent is that by so affecting the RMV it limits its ability to transport the IMPs to the cell surface.

The agent of the invention therefore comprises the following functional Domains;

> Domain B, the Binding Domain, binds the agent to a Binding Site on the target cell capable of undergoing endocytosis to produce an endosome containing the agent
>
> Domain T, the Translocation Domain, translocates the agent or part of the agent from within the endosome across the endosomal membrane into the cytosol of the cell
>
> Domain E, the Effector Domain, inhibits transport of IMPs to the surface of the cell by RMVs.

Domain B can be made to have specificity for a target cell type. The ability to target an agent to a particular cell type is well known in the art. Thus, the functions of Domain B could be achieved by the use of one of many cell-binding molecules known in the art including, but not limited to, antibodies, monoclonal antibodies, antibody fragments (Fab, $F(ab)'_2$, Fv, single chain antibodies, etc.), hormones, cytokines, growth factors and lectins.

The functions of Domain T could be achieved by molecules capable of forming appropriate pores within the endosomal membrane. It is well documented that certain parts of toxin molecules are

capable of forming such pores including, amongst others, fragments of anthrax toxin, botulinum toxin, tetanus toxin, Diphtheria toxin and Pseudomonas endotoxin (Hoch, DH; Romero-Mira, M; Ehrlich, BE; Finkelstein, A; Das Gupta, BR; & Simpson, LL; 1985 PNAS 82 1692 - 1696, Olsnes, S; Stenmark, H; Moskaug, JO; McGill, S; Madshus, IH; & Sandvig, K, 1990, Microbial Pathogenesis 8, 163 - 168.) One such molecule is the Heavy chain of clostridial neurotoxins for example botulinum neurotoxin type A. Preferably it has been found to use only those portions of the toxin molecule capable of pore-forming within the endosomal membrane.

The functions of Domain E, the inhibition of the ability to transport the IMPs to the surface of the cell are not known to the art. Surprisingly, it has been found that different portions of certain toxin molecules - functionally distinct from those capable of pore-formation, including fragments of clostridial neurotoxins, such as either botulinum or tetanus toxins, when introduced into the cytoplasm of target cells are capable of inhibiting the transport of the IMPs in RMVs to the surface of the cell, so reducing the concentration of those IMPs at the cell surface. In particular it has been found that fragments of tetanus toxin and botulinum types A,B,C_1,D, E, F and G are particularly suitable. An example of such a molecule is that portion of a clostridial neurotoxin known as the H_2L fragment, in which the neuronal targeting activity of the carboxyterminal half of the heavy chain of the toxin has been removed, leaving the amino terminal half disulphide - linked to the light chain. Another example would be the Light chain of a clostridial neurotoxin such as the Light chain of the botulinum neurotoxin type B, in particular those portions of the molecule which have Zn^{++} dependent metalloprotease activity.

The invention therefore includes an agent of the following structure;

Domain B--Domain T--Domain E

The Domains are covalently linked by linkages which may include

appropriate spacer regions between the Domains.

In one embodiment of the invention Domain B is a binding molecule capable of binding to a Binding Site on the target cell which undergoes endocytosis, Domain T is the Heavy chain of a botulinum neurotoxin or fragments thereof and Domain E is the Light chain of a botulinum neurotoxin or fragments thereof. Domains T and E can be from the same or different serotypes of *C.botulinum*.

In another embodiment of the invention Domain B is a binding molecule capable of binding to a Binding Site on the target cell which undergoes endocytosis, Domain T is the Heavy chain of a tetanus neurotoxin or fragments thereof and Domain E is the Light chain of a botulinum neurotoxin or fragments thereof.

In another embodiment of the invention Domain B is a binding molecule capable of binding to a Binding Site on the target cell which undergoes endocytosis, Domain T is the Heavy chain of a botulinum neurotoxin or fragments thereof and Domain E is the Light chain of a tetanus neurotoxin or fragments thereof.

In another embodiment of the invention Domain B is a binding molecule capable of binding to a Binding Site on the target cell which undergoes endocytosis, Domain T is the Heavy chain of a tetanus neurotoxin or fragments thereof and Domain E is the Light chain of a tetanus neurotoxin or fragments thereof.

It is to be understood that this invention includes any combination of toxin molecules or fragments of toxin molecules from the same or different organisms which have the functions described.

When the agent is administered to an organism the concentration of IMPs at the surface of the target cell is reduced. This can lead to a number of desired effects including reduced intake of a metabolite or ion into or across the cell, reduction in response of the target cell to a signalling molecule, or change in the immune response of the organism.

EXAMPLES

EXAMPLE 1

3T3-LI fibroblasts are trypsinized into suspension and are electroporated at 300V/ cm, 960 mF with a time constant of 11 - 11.5 msecs, using a Bio-Rad Gene Pulser with capacitance extender, in the presence or absence of 1mM botulinum neurotoxin-B (BoNT-B). Following electroporation the cells are allowed to adhere and are maintained in monolayer culture at 37°C in 24-well plates for 72 h. The cells are then washed and incubated for 5 min at 37°C in the presence or absence of 5nM insulin-like growth factor type 1 (IGF-1), followed by standing on ice for 5 min. The supernatant is aspirated from the cells and replaced with ice-cold 1.5 nM ^{125}I-transferrin (sp. act. 47 Tbq / mmol). Non-specific binding is estimated in parallel incubations performed in the presence of a 100 fold molar excess of non-radioactive transferrin. After 2h the supernatant is removed, and following 3 washes with ice-cold buffer the cell layer is digested in 1N NaOH, and the bound ^{125}I-transferrin measured using a LKB1275 minigamma gamma counter. Up-regulation of transferrin-binding is calculated as the specific ^{125}I-transferrin binding in the presence of IGF -1 expressed as a percentage of the specific binding in the absence of IGF-1.

Table 1 shows that there is a reduced elevation of ^{125}I-transferrin binding in response to IGF-1 in BoNT-B treated cells compared to control. This indicates that introduction of BoNT-B into the cytosol of 3T3-LI fibroblasts inhibits the IGF-1 stimulated up-regulation of transferrin receptors in these cells.

Triton-X-114-soluble proteins extracted from the 3T3-LI fibroblasts digests are analysed by Western blotting using a polyclonal antibody raised against a peptide sequence SEQ.ID.1: (QQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKRKYWWK NLK) identified in a secretory vesicle protein of neurosecretory cells. This anti-vesicle antibody shows reduced reactivity with the relevant doublet band in samples from BoNT-B-treated fibroblasts, which have no reported neurosecretory activity.

Thus, BoNT-B is modifying vesicle (presumably RMV) structure in 3T3-LI fibroblasts concurrently with inhibiting up-regulation of transferrin receptors.

EXAMPLE 2

3T3-LI fibroblasts are electroporated in the presence or absence of 0.5 mM botulinum neurotoxin-A (BoNT-A) using conditions identical to those given in example 1. IGF-1 stimulation of ^{125}I-transferrin binding is assayed in treated and untreated cells as described in example 1.

The results in table 2 show that BoNT-A treatment of 3T3-LI fibroblasts abolishes the up regulation of ^{125}I-transferrin binding seen in response to IGF-1. This indicates that introduction of BoNT-A into the cytosol of 3T3-LI fibroblasts inhibits the IGF-1 stimulated up-regulation of transferrin receptors in these cells.

EXAMPLE 3

3T3-LI fibroblasts are electroporated in the presence or absence of 0.5mM of the H_2L-fragment of BoNT-A (H_2L-A) using conditions identical to those given in example 1. This fragment is produced from the neurotoxin, serotype A, of C botulinum by limited proteolysis using tosylphenylalaninechloromethane-treated trypsin. The H_2L complex is then purified by chromatography on Sephadex G-200 (Shone, CC; Hambleton, P; & Melling, J; 1985, Eur J Biochem 151, 17-82). Electroporation is performed as described in example 1 as is the measurement IGF-1 stimulation of ^{125}I-transferrin binding in treated and untreated cells.

The results in table 3 show that H_2L-A treatment of 3T3-LI fibroblasts inhibits the up-regulation of ^{125}I-transferrin binding seen in response to IGF-1. This indicates that introduction of the H_2L-A fragment of botulinum neurotoxin-A into the cytosol of 3T3-LI fibroblast inhibits the IGF-1 stimulated up-regulation of transferrin receptors in these

cells.

EXAMPLE 4

3T3-LI adipocytes are differentiated from 3T3-LI fibroblasts by treatment with dexamethasone, 3-isobutyl-1-methylxanthine and insulin as described (Frost, SC & Lane MD, 1985, J Biol Chem 260, 2646 -2652). The 3T3-LI adipocytes 7 days after differentiation are treated with Botulinum neurotoxin serotype A diluted into Dulbecco's modified Eagles medium containing serum and filter sterilised (final concentration BoNT A:200nM). Toxin treated and control cells are incubated at 37°C for 45 hours in 8% CO_2. The cells are then washed twice and incubated in 8% CO_2 for 2 hours in serum-free Dulbecco's modified Eagle's medium after which the cells are washed in Krebs Ringer phosphate and incubated in either Krebs Ringer phosphate (basal uptake) or Krebs Ringer phosphate containing 100nM insulin (stimulated uptake) for 15 minutes at 37°C. Glucose uptake is initiated by the addition of [^{3}H] 2-deoxyglucose (14.2KBq, 10uM glucose). After 10 minutes at 37°C the reaction is stopped by aspiration of the glucose solution and rapid washing with ice cold phosphate buffered saline. Cells are lysed by the addition of 0.2N NaOH and the solution neutralised by the addition of 0.2N HCl. Uptake of [^{3}H] 2-deoxyglucose is measured by liquid scintillation counting in optiphase scintillant using a Wallac 1410 liquid scintillation counter.

It is known that clostridial neurotoxins are able to enter certain neurosecretory cells (for example PC12 cells) via a low affinity receptor if high concentrations of the neurotoxin are incubated with the cells for prolonged periods. This process appears to use a pathway via a receptor which is distinct from the highly specific and high affinity receptor present at the neuromuscular junction. Additionally it has been reported that certain clostridial toxins have effects on phagocytic cells, such as macrophages, where entry into the cell is presumed to be via the specific phagocytic activity of these cells. Generally, it is recognised, however, that the neuronal selectivity of clostridial neurotoxins is a result of a very selective binding and cell entry mechanism. It is, therefore,

the surprising finding of these studies, that incubation of 3T3-LI adipocytes with botulinum neurotoxin-A, as described, causes a marked inhibition of insulin-stimulated up-regulation of [^{3}H] 2-deoxyglucose transport (table 4). It is known that insulin-up regulation of glucose transport in adipocytes is a result of movement of glucose transporter proteins from intracellular pools (RMVs) to the cell surface. Thus, this result demonstrates that botulinum neurotoxin-A inhibits the insulin-stimulated movement of glucose transporters in RMVs to the cell surface of adipocytes.

EXAMPLE 5

3T3-LI adipocytes are trypsinised and a suspension of the cells is electroporated in the presence or absence of Botulinum B (0.32mM). A 960 mF capacitor is used for electroporation producing a pulse strength of 300 V/cm; the time constant is 11-12 ms. After electroporation the cells are washed and plated out in a 6 well plate with media and serum. The cells are incubated at 37°C in a humidified atmosphere (air/CO_2; 92.5%/7.5%) for 72 h. At the end of this period, the cells are washed and extracted into 0.1N NaOH. Following neutralisation of the extract with 0.1N HCl the membrane proteins are partitioned into Triton X-114 and subsequently analysed by Western blotting using the anti-vesicle antibody described in example 1. The surprising finding of this study is that electroporation of botulinum neurotoxin into the cytosol of adipocytes results in a modification of vesicle (presumably RMV) structure as evidenced by reduced reactivity of the antibody with the relevant doublet band on samples from botulinum neurotoxin-B treated cells.

EXAMPLE 6

In this example, an agent is synthesized to regulate the cell surface expression of the insulin-dependent glucose transporter of adipocytes.

The binding Domain (B) for the agent in this example is insulin-

like growth factor II, which is purified from the conditioned medium of BRL-3A cells as described (Marquette, H; Todaro, GJ; Henderson, LE & Oroszlan, S, 1981, J Biol. Chem 256 2122-2125).

The translocating Domain (T) is prepared from the neurotoxin, serotype A, of *C. botulinum* by limited proteolysis of the neurotoxin with tosylphenylalaninechloromethane-treated trypsin. The fraction containing Domain T is then purified by chromatography on Sephadex G-200 (Shone, CC; Hambleton, P; & Melling, J; 1985, Eur. J. Biochem. 151, 75-82). This fraction is then applied in phosphate/borate buffer, pH 8.4 onto a quaternary aminoethyl-Sephadex column, and incubated on the column at 4°C overnight with 2M urea and 0.1M dithiothreitol. The column is then washed with buffer containing 2M urea and10mM dithiothreitol. Domain T is then eluted using phosphate/borate buffer containing 2M urea and 10mM dithiothreitol and a stepwise gradient of NaCl from 0.1 to 0.2M (Poulain, B; Wadsworth, JDF; Maisey, EA; Shone, CC; Melling, J; Tauc, L; & Dolly, JO, 1989, Eur. J. Biochem. 185, 197-203). The clostridial neurotoxins are disulphide-linked dichain proteins consisting of a heavy chain and a light chain (Simpson, LL, 1986, Ann. Rev. Pharmicol. Toxicol. 26, 427-453). It should be noted that the Domain T, produced in the manner given, is equivalent to a fraction of the heavy chain of the neurotoxin referred to as H_2.

The effector Domain (E) is prepared from the neurotoxin of *C. tetani* by isoelectric focusing in a sucrose gradient with ampholyte under reducing conditions in 2M urea (Weller, U; Dauzenroth, M-E; Meyer zu Heringdorf, D & Habermann, E, 1989, Eur. J. Biochem., 182, 649-656). It should be noted that the Domain E produced in the manner given is equivalent to the light chain of the neurotoxin, commonly referred to as LC.

Domains E and T are mixed together in equimolar proportions under reducing conditions and covalently coupled by repeated dialysis, at 4°C with agitation, into physiological salt solution in the absence of reducing reagents. Any remaining free sulphydryls are derivatized by the addition of 150mM iodoacetamide for 30 min at 4°C in the dark. The conjugated E-T

product is purified by size exclusion chromatography on Sephadex G-150 using potassium phosphate buffer, pH 7.0. Finally, Domain B is coupled to the E-T complex using N-succinimidyl 3-(2-pyridylothio) proprionate (SPDP). The E-T complex (5 mg) is dissolved in 1 ml of phosphate buffered saline (PBS), and to this is added 200 mg of SPDP dissolved in 0.5 ml of absolute ethanol. After reacting the mixture at room temperature for 30 mins, the 2-pyridyldisulphide-substituted peptide is separated from excess SPDP by gel filtration through Sephadex G25. Domain B is similarly treated, but using less SPDP (20 mg in 0.2 ml ethanol). The substituted Domain B is again harvested from a Sephadex G25 column, and is then reduced by the addition of dithiothreitol to a final concentration of 0.05M. Excess reducing agent is removed by gel filtration on Sephadex G25. Equal portions (w/w) of the substituted E-T complex and the substituted and reduced Domain B are then mixed together and left at 4°C for 18h. The agent is then purified by chromatography on Sephadex G-150 using potassium phosphate buffer, pH 7.0.

The agent, prepared as described, is then tested for its ability to inhibit the insulin-stimulated increase in glucose transporter expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes are differentiated from 3T3-L1 fibroblasts by treatment with dexamethasone, 3-isobutyl-1-methylxanthine and insulin as described (Frost, SC & Lane MD, 1985, J Biol Chem 260, 2646-2652), and are used between 8 and 12 days after initiation of differentiation. Cells are incubated with or without the agent for 90 min at 37°C. Cells are then incubated for 2 hours in serum-free Dulbecco's modified Eagle's medium at the beginning of each experiment. Insulin-treated cells are then exposed for 10 minutes to 10^{-7}M insulin which is added from a stock 1.6 x 10^{-4}M solution. After treatment as described above the cells are washed quickly with Krebs-Ringer phosphate at 37°C and the uptake of [3H] 2-deoxyglucose (14.2 KBq;10 mM) in Krebs Ringer phosphate at 37°C with or without 10^{-7}M insulin over a 10 minute period is then measured. The reaction is stopped by aspiration of the glucose solution and rapid washing with ice cold phosphate buffered saline. Cells are lysed by the addition of

0.2M sodium hydroxide and the solution is neutralised by the addition of 0.2M hydrocholoric acid prior to scintillation counting in Optiphase scintillant using a Wallac 1410 liquid scintillation counter.

EXAMPLE 7

In another example of the invention Domains E and T are produced from the same serotype of botulinum neurotoxin and are produced already coupled together. The neurotoxin, serotype A, of *C. botulinum* is subjected to limited proteolysis using tosylphenylalaninechloromethane-treated trypsin. The E-T complex is then purified by chromatography on Sephadex G-200 (Shone, CC; Hambleton, P; & Melling, J 1985, Eur. J. Biochem. 151, 75-82).

It should be noted that this fragment is equivalent to that referred to as the H_2L fragment. Any remaining free sulphydryls are derivatized by the addition of 150mM iodoacetamide as described in example 6. The binding Domain (B) is insulin-like growth factor II prepared as described in Example 6, and coupled to the E-T complex using SPDP as described. The activity of the agent on the expression of insulin-dependent glucose transport in adipocytes is tested as described in Example 6.

EXAMPLE 8

In another example of the invention, an agent for the regulation of the cell surface expression of the CR1 receptor for complement fragment C3b in neutrophils (CD 35) is synthesized in the following manner. The B Domain is prepared from the SHCL3 monoclonal antibody to the leukocyte adhesion molecule P150,95. The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 7, and are coupled to Domain B, as described in that example.

The preferred method for testing the activity of the agent on neutrophil cell surface expression of CR1 (CD35) is using the whole blood lysing technique. EDTA anticoagulated whole blood

from normal donors is treated with the agent for 4 hours and then activated for 30 minutes at 37°C using 10^{-6}M fMet-Leu-Phe diluted in PBS from a stock of 10^{-2}M made up in DMSO. Control cells are incubated with PBS. The blood is then incubated for 30 minutes at room temperature with 10ml of Phycoerythrin conjugated monoclonal antibody antiCD35 (Serotec:MCA 554PE), red blood cells are lysed using Becton Dickinson lysing fluid, leukocytes washed with PBS and resuspended in 2% formaldehyde in PBS. Suface bound PE is analysed by flow cytometry using a FACScan (Becton Dickinson) equipped with Lysys II software.

EXAMPLE 9

In another example of the invention, an agent for the regulation of the cell surface expression of the leukocyte adhesion molecule Mac-1 (CD IIb) is synthesized in the following manner. The B Domain is prepared from the SHCL3 monoclonal antibody to the leukocyte adhesion molecule P150,95 by standard methodologies using pepsin, and is purified by gel filtration (Martin, FJ; Hubbell, WL; and Papahadjopoulos, D, 1981, Biochemistry 20, 4229-4238). The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 7, and are coupled to Domain B as described in that example.

The preferred method for testing the activity of the agent on neutrophil cell surface expression of Mac-1 (CD11b) is using the whole blood lysing technique. EDTA anticoagulated whole blood from normal donors is treated with the agent for 4 hours at 37°C and then activated for 30 minutes at 37°C using 10^{-6}M fMet-Leu-Phe diluted in PBS from a stock of 10^{-2}M made up in DMSO. Control cells are incubated with PBS. The blood is then incubated for 30 minutes at room temperature with 10ml of fluoroscein isothiocyanate conjugated monoclonal antibody antiCD11b (Serotec:MCA 551F). The red blood cells are lysed using Becton Dickinson lysing fluid, the leukocytes washed with PBS and resuspended in 2% formaldehyde in PBS. Surface bound FITC is analysed by flow cytometry using a FACScan (Becton Dickinson) equipped with Lysys II software.

EXAMPLE 10

In another example of the invention, an agent for the regulation of the cell surface content of Na^+ channels in the apical membrane of bladder epithelium is synthesized in the following manner. The B Domain is prepared from a high affinity monoclonal antibody to a cell surface marker of bladder epithelial cells by standard methodology using pepsin, and is purified by gel filtration (Martin, FJ; Hubbell, WL; and Papahadjopoulos, D, 1981, Biochemistry 20, 4229-4238). The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 7, and are coupled to Domain B as described in that example.

The effect of the agent on aldosterone-stimulated increases in amiloride-sensitive Na^+- channels is tested using urinary epithelial cells. Bladder epithelial cells, prepared as primary cultures from rat bladder (Johnson, MD; Bryan, GT; Reznikoff, CA;1985, J Urol 133, 1076-1081), are incubated with or without the agent for 90 mins at 37°C. Aldosterone-treated cells are then exposed for 1h to aldosterone. After treatment as described, the cells are rapidly washed and the amiloride-sensitive uptake of $^{22}Na^+$ over a 5 min incubation at 37°C is measured.

EXAMPLE 11

In another example of the invention, an agent for regulating antigen-presentation by B-cells is synthesized in the following manner. The B Domain is prepared from the pan B cell monoclonal antibody LL2 using standard methodology using pepsin, and is purified by gel filtration (Martin, FJ; Hubbell, WL & Papahadjopoulos, D, 1981, Biochemistry, 20, 4229-4238). The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 3, and are coupled to Domain B also as described in that example.

The effect of the agent on antigen-presentation is tested using the murine B lymphoma cell-line TA3. These cells are first

incubated with the agent for 90 mins at 37°C, and then hen egg lysozyme (HEL) is added and the incubation continued for 2h at 37°C. The TA3 cells are then fixed and washed before culture with the I-A^k- restricted HEL46-61 specific T-cell hybridoma 3A9 (Lorenz, RG & Allen, PM, 1989, Nature 337, 560). The supernatant from the 3A9 cells is tested for its ability to support growth of the IL-2-dependent cell line, CTLL. Proliferative responses are measured by the incorporation of ^{3}H-thymidine over a 3h period following 2 days of culture with the supernatant.

The examples described above are purely illustrative of the invention. It should be clear to those skilled in the art that any combination of the three domains are within the scope of this invention. In synthesising the agent the coupling of the T-E component of the invention to the targeting component is achieved via chemical coupling using reagents and techniques known to those skilled in the art. Thus, although the examples given use exclusively the SPDP coupling reagent any other coupling reagent capable of covalently attaching the targeting component of the reagent and known to those skilled in the art is covered in the scope of this application. Similarly it is evident to those skilled in the art that either the DNA coding for either the entire agent or fragments of the agent could be readily constructed and, when expressed in an appropriate organism, could be used to produce the agent or fragments of the agent. Such genetic constructs of the agent of the invention obtained by techniques known to those skilled in the art are also covered in the scope of this invention.

EXPLOITATION IN INDUSTRY

The agent described in this invention can be used *in vivo* either directly or as a pharmaceutically acceptable salt or ester in a method of treatment for a variety of pathophysiological states.

For example, one form of the agent can be used in a method of treatment for glucose metabolism disorders by limiting the

uptake of glucose by certain cells. A specific example of this would be the use of a form of the agent in a method of treatment for clinical obesity by limiting the uptake of glucose by adipose cells and hence reducing accumulation of lipid in these cells.

In another example a form of the agent can be used in a method of treatment for hypertension by regulating the ion uptake by kidney cells and hence controlling the output of fluid from these organs.

In yet another example a form of the agent can be used in a method of treatment for inflammation by controlling the response of target cells to external signals which trigger the inflammatory response.

In yet another example a form of the agent can be used in a method of treatment for immune disorders by controlling the presentation of peptide sequences by antigen presenting cells to the effector cells of the immune system.

TABLE 1

IGF-1 Up-Regulation Of ^{125}I-Transferrin Binding In 3T3-LI Fibroblasts

Treatment	IGF-1	% basal binding ± SD*
Control	-	100 ± 28 (n=3)
	+	258 ± 46 (n=3)
BoNT-B	-	100 ± 8 (n=3)
	+	149 ± 27 (n=3)

* Specific binding of ^{125}I-transferrin to 3T3-LI fibroblasts expressed as a percentage of the specific binding in the absence of IGF-1.

TABLE 2

IGF-1 Up-regulation of ^{125}I-transferrin binding in 3T3-LI fibroblasts

Treatment	IGF-1	% basal binding ± SD*
Control	-	100 ± 28 (n=3)
	+	258 ± 46 (n=3)
BoNT-A	-	100 ± 44 (n=3)
	+	149 ± 10 (n=3)

* Specific binding of ^{125}I-transferrin to 3T3-LI fibroblasts expressed as a percentage of the specific binding in the absence of IGF-1.

TABLE 3

IGF-1 Up regulation of ^{125}I-transferrin binding in 3T3-LI fibroblasts

Treatment	IGF-1	% basal binding ± SD*
Control	-	100 ± 28 (n=3)
	+	258 ± 46 (n=3)
H_2L-A	-	100 ± 15 (n=3)
	+	134 ± 60 (n=3)

* Specific binding of ^{125}I-transferrin to 3T3-LI fibroblasts expressed as a percentage of the specific binding in the absence of IGF-1.

TABLE 4

Uptake of [^{3}H] -2-deoxyglucose by 3T3-LI adipocytes

	Basal	Insulin-stimulated
Control	1655 ± 67 (n=3)	14 328 ± 264 (n=3)
BoNT-A treated	2306 ± 49 (n=3)	5587 ± 322 (n=3)

The results are the means ± SEM of triplicate determinations and are given as the total dpm taken up by the cell monolayer during a 10 min incubation.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: NOVEL AGENT FOR CONTROLLING CELL ACTIVITY

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
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(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9305735.4
(B) FILING DATE: 19-MAR-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
1 5 10 15

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp
20 25 30

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser
35 40 45

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys
50 55 60

CLAIMS

1. An agent for controlling the interaction of a cell with its external environment by controlling the transport of Integral Membrane Proteins to the membrane of the cell in Recyclable Membrane Vesicles.

2. An agent according to Claim 1 for the control of the level of the IMP responsible for the transport of a metabolite across the cell membrane, so controlling the availability of that metabolite within the cell.

3. An agent according to Claim 1 for the control of the level of the IMP responsible for the transport of a metabolite across the cell membrane into the cell and out of the cell, so controlling the transport of that metabolite through the cell.

4. An agent according to Claim 1 for the control of the level of the IMP responsible for the selective permeability of the plasma membrane of the cell to an ion, so modulating the concentration of that ion within the cell.

5. An agent according to Claim 1 for the control of the level of the IMP responsible for the recognition of a signalling molecule, so modulating the responsiveness of the cell to that signalling molecule.

6. An agent according to Claim 1 for the control of the level of the IMP responsible for the transduction of signals across the cell membrane following binding to the membrane of a signalling molecule, so modulating the responsiveness of the cell to that signalling molecule.

7. An agent according to Claim 1 for the control of the

level of the IMP responsible for the display on the cell surface of peptide fragments derived from ingested antigens.

8. An agent according to any preceding claim which has target specificity for target cell types so that the scope of the effect of the agent is limited to said cell types.

9. An agent according to any preceeding claim which comprises three Domains B,T and E linked together in the following manner:

Domain B--Domain T--Domain E

Where

Domain B is the Binding Domain which binds the agent to a Binding Site on the cell which undergoes endocytosis to produce an endosome

Domain T is the Translocation Domain which translocates the agent (with or without the Binding Site) from within the endosome across the endosomal membrane into the cytosol of the cell

Domain E is the Effector Domain which inhibits the ability of the RMVs to transport the IMPs to the surface of the cell.

10. An agent according to Claim 9 which comprises three Domains B,T and E linked together in the following manner;

Domain B--X--Domain T--X--Domain E

Where X is either a spacer molecule or a covalent linkage but at least one X is a spacer molecule.

11. An agent according to Claims 9 or 10 in which Domain B, the Binding Domain, binds to a Binding Site which is characteristic of a particular cell type.

12. The agent according to any preceding claim in which

Domain B comprises a monoclonal antibody to a surface antigen on the target cell capable of undergoing endocytosis to produce an endosome
Domain T comprises a domain or domain fragment of a toxin molecule which translocates the agent from the resulting endosome into the cytosol of the cell
Domain E comprises a different domain or domain fragment of a toxin molecule functionally distinct from Domain T with Zn^{++} dependent proteolytic activity.

13. The agent according to any preceding claim in which

Domain B comprises a ligand to a cell surface receptor on the target cell capable of undergoing endocytosis to produce an endosome
Domain T comprises a domain or domain fragment of a toxin molecule which translocates the agent from the resulting endosome into the cytosol of the cell
Domain E comprises a different domain or domain fragment of a toxin molecule functionally distinct from Domain T with Zn^{++} dependent proteolytic activity.

14. An agent according to claim 13 which affects the rate of glucose uptake by adipose cells in response to insulin in which

Domain B is a ligand to the insulin-like growth factor II receptor
Domain T is the domain or domain fragment of the botulinum neurotoxin Heavy chain responsible for translocation of the toxin across the cell membrane
Domain E is the domain or domain fragment of the Light chain of botulinum neurotoxin having Zn^{++} dependent metalloprotease activity.

15. An agent according to any of claims 9 to 14 in which Domains T and E are obtained from Clostridial

neurotoxin.

16. A process for the manufacture of an agent according to any preceding claim which comprises the covalent attachment of three Domains B,T and E in the following manner;

Domain B--Domain T--Domain E

Where
Domain B is the Binding Domain which binds the agent to a Binding Site on the cell which undergoes endocytosis to produce an endosome
Domain T is the Translocation Domain which translocates the agent (with or without the Binding Site) from within the endosome across the endosomal membrane into the cytosol of the cell
Domain E is the Effector Domain which inhibits the ability of the RMVs to transport the IMPs to the surface of the cell.

17. A process for the manufacture of an agent according to Claim 16 which comprises the covalent attachment of three Domains B,T and E in the following manner;

Domain B--X--Domain T--X--Domain E

Where X is either a spacer molecule or a covalent linkage but at least one X is a spacer molecule.

18. A process according to Claims 16 or 17 in which Domain B, the Binding Domain, binds to a Binding Site which is characteristic of a particular cell type.

19. A method of making the agent according to any preceding claim comprising constructing a genetic construct which codes for the agent, incorporating said construct into a host organism and expressing

the construct to produce the agent.

20. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of glucose metabolism disorders.

21. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of clinical obesity.

22. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of hypertension.

23. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of inflammatory disorders.

24. Use of the agent or a pharmaceutically acceptable salt thereof according to any preceding claim for the treatment of immune system disorders.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00558

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K47/48 C07K15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 00099 (FOSKNINGSSTIFTELSEN DET NORSKE RADIUMHOSPITAL) 9 January 1992 see page 3, line 2 - line 14; claim 1 ---	1-24
A	WO,A,93 04191 (NEORX CORPORATION) 4 March 1993 see page 25, paragraph 2; claims 11,2,4,8 ---	1-24
A	WO,A,91 17173 (CYTOGEN CORPORATION) 14 November 1991 see claim 1 --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
12 August 1994	

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00558

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	BIOCHEMISTRY, vol.33, 1994, EASTON, PA US pages 2604 - 2609 SIMS K. KOCHI ET AL. 'THE EFFECTS OF PH ON THE INTERACTION OF ANTHRAX TOXIN LETHAL AND EDAMA FACTORS WITH PHOSPHOLIPID VESICLES' See abstract -----	9-24

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB94/00558

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
please see enclosure!

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International lication No
PCT/GB 94/00558

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9200099	09-01-92	AU-A-	8000191	23-01-92
		CA-A-	2086342	28-12-91
		EP-A-	0542756	26-05-93
		JP-T-	6503552	21-04-94
WO-A-9304191	04-03-93	NONE		
WO-A-9117173	14-11-91	US-A-	5196510	23-03-93
		EP-A-	0527954	24-02-93

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(30) Priority Data:
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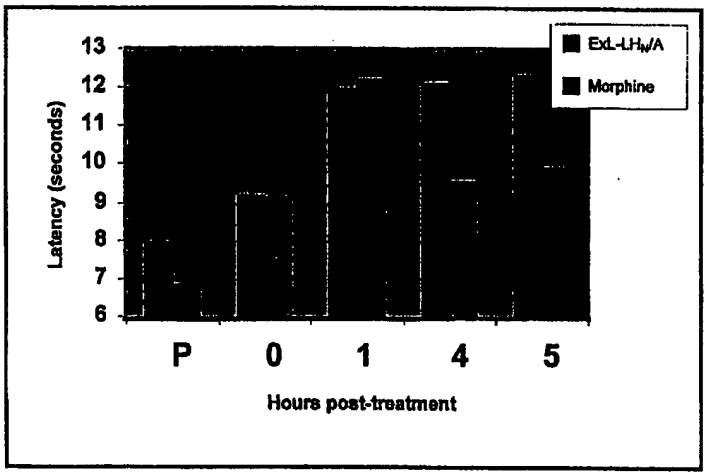
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CONJUGATES OF GALACTOSE-BINDING LECTINS AND CLOSTRIDIAL NEUROTOXINS AS ANALGESICS



(57) Abstract

A class of novel agents that are able to modify nociceptive afferent function is provided. The agents may inhibit the release of neurotransmitters from discrete populations of neurones and thereby reduce or preferably prevent the transmission of afferent pain signals from peripheral to central pain fibres. They comprise a galastose-binding lectin linked to a derivative of a clostridial neurotoxin. The derivative of the clostridial neurotoxin comprises the L-chain, or a fragment thereof, which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity. The agents may be used in or as pharmaceuticals for the treatment of pain, particularly chronic pain.

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EE	Estonia	LR	Liberia	SG	Singapore		

Conjugates of galactose-binding lectins and clostridial neurotoxins as analgesics.

Technical field

This invention relates to a class of novel agents that are able to modify nociceptive afferent function. The agents may inhibit the release of neurotransmitters from discrete populations of neurones and thereby reduce or preferably prevent the transmission of afferent pain signals from peripheral to central pain fibres. The agent may be used in or as a pharmaceutical for the treatment of pain, particularly chronic pain.

Background

The sensation of pain due to injury or disease is carried from the periphery to the brain by a multi-neuronal pathway. The first part of this system comprises the primary nociceptive afferents that form synapses with secondary neurones in the dorsal horn of the spinal cord, or the nuclei of the cranial nerves. These synapses pass on the incoming information by the release of neurotransmitters and neuromodulators such as glutamate and substance P. These synapses are, therefore, possible sites for intervention to alleviate pain, indeed one of the modes of action of the opiate analgesics is to down-modulate neurotransmitter release at these synapses.

Unfortunately, the opiates have a number of limitations as drugs. Firstly, there are a number of chronic pain conditions for which the opiates are not effective. Secondly, the opiates have a number of side effects that are mediated both peripherally (constipation) and centrally (respiratory depression and euphoria) which present problems for long term use.

There is, therefore, a need for the development of new pharmaceuticals for the treatment of pain, particularly chronic pain.

One approach to this problem is the use of new agents containing fragments of clostridial neurotoxins (WO96/33273).

The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of bacterium of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight different classes of the neurotoxins known: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C_1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesised by the host bacterium as single polypeptides that are modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), which has a molecular mass of approximately 100 kDa, and the light chain (L), which has a molecular mass of approximately 50 kDa.
Two distinct functions can be identified within the H-chain; binding and translocation. The carboxy-terminal half (H_C) is involved in the high affinity, neurospecific binding of the toxin to cell surface acceptors, whilst the amino-terminal half (H_N) is central to the translocation of the toxin into the neuronal cell. For botulinum neurotoxin type A these domains are considered to reside within amino acid residues 872-1296 for the H_C, amino acid residues 449-871 for the H_N and residues 1-448 for the LC. The minimal domains necessary for the activity of the light chain of clostridial toxins are described in J. Biol. Chem. Vol.267, No.21, July 1992, pages 14721-14729. The eight distinct neurotoxin light chains (L) are highly specific zinc-dependent endopeptidases which each

hydrolyse different but specific peptide bonds in one of three substrate proteins, synaptobrevin, syntaxin or SNAP-25. These substrates are important components of the neurosecretory machinery. The hydrolytic activity of the clostridial toxins results in a prolonged muscular paralysis. The functions of all three identified domains are necessary for the toxic activity of the clostridial endopeptidases.

Some of the clostridial endopeptidases, most notably botulinum neurotoxin type A, have been used as pharmaceutical agents for the treatment of a range of muscle dystonias. The flaccid paralysing action of the native botulinum toxins makes them appropriate for this use.

The use of fragments of clostridial neurotoxins for the desired purpose of analgesia is dependent on the invention of conjugates, or derivatives of these molecules, with a specific binding activity that will deliver the L-chain endopeptidase to the nociceptive afferent neurons in preference to other neurones in the relevant anatomical locus. Delivery of these conjugates includes binding to the cell surface, internalisation via an endosomal compartment and translocation of the clostridial endopeptidase activity into the cytosol.

Targeting of extracellular species to specific intracellular locations following endocytosis involves an appreciation of a number of possible targeting strategies. It is understood that early endosomes are part of the key sorting mechanisms of the cell, routing species to late endosome (and onto lysosomes for degradation), recycling to the cell surface or to the Trans-Golgi Network. Intracellular routing determinants have been suggested that determine the pathway and final destination of

particular species (Mellman, 1996, Annu. Rev. Cell Biol., 12, 575-625).

Current data suggests that translocation of native clostridial neurotoxins occurs from an acidic intracellular compartment, though the exact location and nature of the compartment is unknown (Montecucco & Schiavo, 1994, Mol. Micro. 13, 1-8). In patent WO96/33273 it is proposed that for an agent to be effective, the agent must target to an appropriate compartment for translocation of the toxin. As an example of specific intracellular targeting, internalisation of the NGF-receptor is by specific endocytosis and retrograde routing (initiated by receptor-ligand complex), via acidic endosomes to the cell body, and an agent incorporating NGF is given in support of WO96/33273.

Statement of Invention

The present invention relates to an agent that can reduce and preferably prevent the transmission of pain signals from the periphery to the central nervous system, thereby alleviating the sensation of pain. Specifically, the invention can provide an agent that can reduce and preferably prevent the transmission of pain signals from nociceptive afferents to projection neurones. More specifically, the invention can provide an agent that can inhibit the exocytosis of at least one neurotransmitter or neuromodulator substance from at least one category of nociceptive afferents.

In one aspect of the invention, an agent is provided which can be administered to the spinal cord, and which can inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents terminating in that region of the spinal cord.

In a second aspect of the invention, there is provided an agent which can specifically target defined populations of afferent neurones, so that the effect of the agent is limited to that cell type.

In a third aspect of the invention, there is provided a method of treatment of pain that comprises administering an effective dose of the agent according to the invention.

In a fourth aspect of the invention, the agent can be expressed recombinantly as a fusion protein that includes the required components of the agent.

Definitions

Without wishing to be limited by the definitions set down, it is intended in this description that the following terms have the following meanings:

Light chain means the smaller of the two polypeptide components of any of the clostridial neurotoxins. It is commonly referred to as the L-chain or simply L. An L-chain has a molecular mass of approximately 50 kDa, and it is a metalloprotease exhibiting high substrate specificity for vesicle and/or plasma membrane associated proteins involved in the exocytotic process.

Heavy chain means the larger of the two polypeptide components of any of the clostridial neurotoxins. It is commonly referred to as H-chain or simply H and has a molecular mass of approximately 100 kDa.

H_c fragment means a peptide derived from the H-chain of a clostridial neurotoxin which is responsible for binding of the native holotoxin to cell surface acceptor(s) involved in the intoxicating action of clostridial toxin prior to

internalisation of the toxin into the cell. It may be approximately equivalent to the carboxy-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain.

H_N fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. It is characterised as:

A portion of the H-chain which enables translocation of that portion of the neurotoxin molecule such that a functional expression of light chain activity occurs within a target cell.

The domain responsible for translocation of the endopeptidase activity, following binding of neurotoxin to its specific cell surface receptor via the binding domain, into the target cell.

The domain responsible for formation of ion-permeable pores in lipid membranes under conditions of low pH.

The domain responsible for increasing the solubility of the entire polypeptide compared to the solubility of light chain alone.

LH_N means a fragment derived from a clostridial neurotoxin that contains the L-chain, or a functional fragment thereof, coupled to a H_N fragment.

BoNT/A means botulinum neurotoxin serotype A, and is a neurotoxin produced by *Clostridium botulinum;* it has a molecular mass of approximately 150kDa.

LH_N/A is LH_N that is derived from *Clostridium botulinum* neurotoxin type A.

Targeting Moiety (TM) means any chemical structure of an agent which functionally interacts with a binding site

causing a physical association between the agent and the surface of a primary sensory afferent.

Primary sensory afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system.

Primary nociceptive afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system, where that information can result in a sensation of pain.

Lectin is any protein that binds to oligosaccharide structures.

Galactose-binding lectin is a lectin that binds to oligosaccharide structures in which the terminal residue is derived from galactose or N-acetylgalactosamine.

Detailed Description of the Invention

It can be seen from this disclosure that an agent for reducing or preventing the transmission of pain signals from peripheral, nociceptive afferent neurones to projection neurones has many potential applications in the reduction of the sensation of pain, particularly of severe chronic pain.

Lectins are a class of proteins, often glycoproteins, that bind to carbohydrate structures. Lectins are found across the whole range of life forms from viruses to mammals. The most commonly exploited sources are the abundant lectins found in the seeds of plants. Lectins have previously been labelled and used as cell surface markers.

According to the invention, there is provided an agent that can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents.

It is known that such an agent can be produced based on the use of fragments of clostridial neurotoxin conjugated to a targeting ligand (WO96/33273). Given the known complexity of intracellular transport and the constraints on construct requirements, it is surprising that conjugates between toxin fragments and a specific sub-class of lectins that bind only to galactosyl residues form agents to produce analgesics that are particularly potent and selective. Inventions incorporating such lectins are the subject of this disclosure and several examples are provided.

One example of a class of plant-derived, galactose-binding lectins are those that can be purified from the seeds of the genus *Erythrina*. These lectins have been characterised to exist predominantly as non-covalent dimeric proteins with total molecular weights of approximately 60 kDa. Lectins have been isolated from several *Erythrina* species including: *E. corallodendron* (Gilboa-Garber and Mizrahi, 1981, Can. J. Biochem. 59, 315-320), *E. cristagalli* (Iglesias et al., 1982, Eur. J. Biochem. 123, 247-252), *E. indica* (Horejsi et al., 1980, Biochim. Biophys. Acta 623, 439-448), *E. arborescens*, *E suberosa*, *E. lithosperma* (Bhattacharyya et al., 1981, Archiv. Biochem. Biophys. 211, 459-470) *E. caffra*, *E. flabelliformis*, *E. latissima*, *E. lysistemon*, *E. humeana*, *E. perrieri*, *E. stricta*, and *E. zeyheri* (Lis et al., 1985, Phytochem. 24, 2803-2809).

These lectins have been analysed for their selectivity for saccharide binding (see e.g. Kaladas et al., 1982, Archiv. Biochem. Biophys. 217, 624-637). They have been found to

bind preferentially to oligosaccharides with a terminal β-D-galactosyl residue.

A second example of a plant-derived, galactose-binding lectin with the desired binding specificity can be obtained from *Glycine max* (soy) beans. This lectin (soya bean agglutinin, SBA) is a tetrameric protein with a total molecular weight of approximately 110 kDa. It binds to oligosaccharides containing galactose or N-acetylgalactosamine residues.

An example of a galactose-binding lectin from bacteria is PA-I, obtained from *Pseudomonas aeruginosa*. PA-I is a D-galactosephilic lectin with a molecular weight of about 13 kDa and it binds to galactose-containing oligosaccharides (Gilboa-Garber and Mizrahi, 1981, Can. J. Biochem. 59, 315-320).

These and other lectins of the sub-class of galactose-binding lectins can be used as targeting moieties (TM) for conjugates of the type described in WO96/33273. The requirements for TMs in these agents are that they show specificity for the primary sensory afferents over other spinal nerves and that they lead to the internalisation of the agents into an appropriate intracellular compartment. The lectins of this invention fulfil these criteria. Surprisingly, in comparison to other lectins of WO96/33273, they can fulfil these criteria more efficiently and can provide agents with enhanced selectivity for nociceptive afferent neurosecretion.

Thus, in one embodiment of the invention a galactose-binding lectin is conjugated, using linkages that may include one or more spacer regions, to a derivative of the clostridial neurotoxins.

In another embodiment of the invention the agent is expressed in a recombinant form as a fusion protein. The fusion protein may be derived from nucleic acid encoding an appropriate fragment of a galactose-binding lectin, in addition to any desired spacer domains, with nucleic acid encoding all or part of a polypeptide of one serotype of neurotoxin. Such a nucleic acid may be a chimera derived from the nucleic acid encoding polypeptides from more than one serotype.

In another embodiment of the invention the required LH_N, which may be a hybrid of an L and H_N from different clostridial toxin serotypes, is expressed as a recombinant fusion protein with the galactose-binding lectin, and may also include one or more spacer regions.

In a further embodiment of the invention the required TM, L or LH_N and translocation domain components may be separately expressed in a recombinant form and subsequently linked, covalently or non-covalently, to provide the desired agent.

In a further embodiment of the invention the required translocation domain may be of a non-clostridial origin, comprising instead a peptide or other entity capable of similar or enhanced function. Examples would include, but not be restricted to, the translocation domain of diphtheria toxin (O'Keefe *et al.*, Proc. Natl. Acad. Sci. USA (1992) **89**, 6202-6206 ; Silverman *et al.*, J. Biol. Chem. (1993) **269**, 22524-22532), the translocation domain of *Pseudomonas* exotoxin type A (Prior *et al.* Biochemistry (1992) **31**, 3555-3559), the translocation domains of anthrax toxin (Blanke *et al.* Proc. Natl. Acad. Sci. USA (1996) **93**, 8437-8442) and a variety of fusogenic or hydrophobic peptides of translocating function (Plank *et al.* J. Biol. Chem. (1994) **269**, 12918-12924).

Exploitation in Industry

The agent described in this invention can be used *in vivo*, either directly or as a pharmaceutically acceptable salt, for treatment of pain.

For example, an agent according to the invention can be administered by spinal injection (epidural or intrathecal) at the level of the spinal segment involved in the innervation of an affected organ for the treatment of pain. This is, for example, applicable in the treatment of deep tissue pain, such as chronic malignant pain.

The present invention will now be described by reference to the following examples together with the Figures that show the following:

Figure 1. SDS-PAGE analysis of fractions from ExL-LH_N/A purification scheme

Figure 2. Cleavage of SNAP-25 by ExL-LH_N/A

Figure 3. SDS-PAGE analysis of fractions from EcL-LH_N/A purification scheme

Figure 4 SDS-PAGE analysis of fractions from SBA-LH_N/A purification scheme

Figure 5 Native gel analysis of ExL- and SBA-LH_N/A

Figure 6 Activity of ExL-LH_N/A on release of neurotransmitter from eDRG and eSC neurons

Figure 7 Activity of SBA-LH_N/A on release of neurotransmitter from eDRG and eSC neurons

Figure 8 Activity of WGA-LH_N/A on release of neurotransmitter from eDRG and eSC neurons

Figure 9 Activity of ExL-LH_N/A in an *in vivo* electrophysiology model of analgesia

Figure 10 Activity of ExL-LH_N/A in an *in vivo* behavioural model of analgesia

Example 1. The Production of a conjugate between a lectin from *Erythrina cristagalli* and LH_N/A.

Materials

Lectin from *E. cristagalli* (ExL) was obtained from Sigma Ltd.

LH_N/A was prepared essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* **167**, 175-180.

SPDP was from Pierce Chemical Co.

PD-10 desalting columns were from Pharmacia.

Dimethylsulphoxide (DMSO) was kept anhydrous by storage over a molecular sieve.

Denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using gels and reagents from Novex

Immobilised lactose-agarose was obtained from Sigma Ltd.

Additional reagents were obtained from Sigma Ltd.

Methods

The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to a final concentration of 10 mg/ml. Aliquots of this solution were stored at -20°C until use.

The ExL was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS over a PD-10 column.

The thiopyridone leaving group was removed from the product by reduction with dithiothreitol (DTT, 5 mM, 30 min). The product of this reaction was analysed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatisation achieved. The degree of derivatisation achieved was 0.8±0.06 mol/mol. The thiopyridone and DTT were removed by once again desalting into PBS over a PD-10 column.

The LH_N/A was desalted into PBSE (PBS containing 1 mM EDTA). The resulting solution (0.5-1.0 mg/ml) was reacted with a four- or five-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO. After 3 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBS.

A portion of the derivatised LH_N/A was removed from the solution and reduced with DTT (5 mM, 30 min). This sample was analysed spectrophotometrically at 280 mm and 343 nm to determine the degree of derivatisation. The degree of derivatisation achieved was 2.26±0.10 mol/mol.

The bulk of the derivatised LH_N/A and the derivatised ExL were mixed in proportions such that the ExL was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for >16 h at 4°C.

The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 10000-50000 molecular weight exclusion limit) prior to a two step purification strategy. As the first step, the concentrated material was applied to a Superose 12 column on an FPLC chromatography system (Pharmacia). The column

was eluted with PBS and the elution profile followed at 280 nm.

Fractions were analysed by SDS-PAGE on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie Blue. The major band of conjugate has an apparent molecular mass of between 130-160 kDa; this is separated from the bulk of the remaining unconjugated LH_N/A and more completely from the unconjugated ExL. Fractions containing conjugate were pooled prior to the second chromatography step; immobilised lactose-agarose. Selected post-Superose-12 fractions were applied to PBS-washed lactose-agarose and incubated for 2 hours at 4°C to facilitate binding. Lectin-containing proteins (i.e. ExL-LH_N/A conjugate) remained bound to the agarose during subsequent washing with PBS to remove contaminants (predominantly unconjugated LH_N/A). ExL-LH_N/A conjugate was eluted from the column by the addition of 0.3M lactose (in PBS) and the elution profile followed at 280 nm. The fractions containing conjugate were pooled, dialysed against PBS, and stored at 4°C until use.

In figure 1 is illustrated the SDS-PAGE profile during different stages in the conjugate purification scheme. Lanes 2 and 3 indicate ExL lectin and LH_N/A respectively prior to conjugation. Lanes 4, 5 & 6 represent conjugation mixture, post-Superose-12 and post-lactose affinity chromatography samples respectively. Lane 6 is therefore indicative of the profile of the final conjugate material. Molecular weight markers are represented in lanes 1 & 7 with sizes indicated on the figure.

On the SDS-PAGE gel there are bands due to lectin alone in fractions containing the conjugate, this material is probably due to the non-covalent homo-dimeric nature of the ExL; where only one monomer of ExL is covalently attached to the LH_N/A the other is dissociated from the complex by the SDS in the electrophoretic procedure giving rise to these bands. The absence of free lectin monomers

was confirmed by native PAGE analysis and is illustrated in Figure 5. ExL-LH_N/A (lane 5) was analysed by non-denaturing PAGE. Samples were separated using 4-20% polyacrylamide gel for 6.75 hours, 125V, 4°C. The electrophoresis profile was compared to those of LH_N/A (lane 3) and ExL lectin only (lane 4). A range of marker proteins were analysed alongside; apoferritin (lane 6), β-amylase (lane 8), alcohol dehydrogenase (lane 7) and albumin (lane 9). Approximate molecular sizes are indicated.

Example 2. The production of a conjugate between a lectin from *Erythrina corallodendron* and LH_N/A.

The procedure for production of a conjugate between a lectin from *Erythrina corallodendron* and LH_N/A is essentially as described in Example 1 but with the following differences:

Materials

Lectin from *E. corallodendron* (EcL) was obtained from Sigma Ltd.

Figure 3 illustrates the purification scheme for the EcL-LH_N/A conjugate. Samples were applied to 4-20% polyacrylamide gradient gels and subjected to electrophoresis prior to staining with Coomassie blue. Lane 1 = molecular weight markers. Lane 2 represents the post-lactose affinity purified sample of EcL-LH_N/A. Lane 3 is a sample of pre-lactose affinity purified (size-exclusion chromatography only) EcL-LH_N/A. Lane 4 is a sample of pre-lactose affinity purified ExL-LH_N/A.

Example 3. The Production of a conjugate between a lectin from *Glycine max* and LH_N/A

The procedure for production of a conjugate between a lectin from *Glycine max*_and LH_N/A is essentially as described in Example 1 but with the following differences:

Materials

Lectin from *G. max* (SBA) was obtained from Sigma Ltd.

Method

For the affinity chromatography step an immobilised N-acetylgalactosamine (GalNAc) column was used and specific SBA-LH_N/A was eluted by the addition of 0.3M lactose. Figure 4 illustrates SDS-PAGE profile changes during the purification scheme for SBA-LH_N/A. SBA-LH_N/A was purified from crude conjugate mixture by Superose-12 size-exclusion chromatography and immobilised N-acetylgalactosamine affinity chromatography. Samples were subjected to SDS-PAGE on 4-20% polyacrylamide gels. Lanes 6-8 were run in the presence of 0.1M DTT. Lanes 1 (&7) and 2 (&8) indicate SBA and SPDP-derivatised LH_N/A respectively, prior to conjugation. Lanes 3, 4 & 5 (&6) represent conjugation mixture, post-Superose-12 and post-affinity chromatography samples respectively. Lane 5 is therefore indicative of the profile of the final conjugate material. Molecular weight markers are represented in lanes Mr with sizes indicated on the figure.

The absence of free lectin monomers was confirmed by native non-denaturing PAGE analysis as illustrated in Figure 5. Samples were separated using 4-20% polyacrylamide gel for 6.75 hours, 125V, 4°C. The electrophoresis profile of SBA-LH_N/A (lane 1) was compared to those of SBA lectin only (lane 2) and LH_N/A (lane 3). A range of marker proteins were analysed alongside; apoferritin (lane 6), β-amylase (lane 8), alcohol dehydrogenase (lane 7) and albumin (lane 9). Approximate molecular sizes are indicated.

Example 4. Activity of ExL-LH_N/A in primary neuronal cultures

The dorsal root ganglia contain the cell bodies of primary nociceptive afferent neurons. It is well established that in primary *in vitro* cultures of this tissue the neurons retain many of the characteristics of the nociceptive afferent. These characteristics include the ability to release neuropeptides such as substance P in response to chemical stimuli known to cause pain *in vivo* (e.g. capsaicin). Neurons anatomically adjacent to those of the DRG include those of the spinal cord. Cultures of SC neurons prepared from embryonic rats can be established *in vitro* and the release of neurotransmitter (^{3}H-glycine) under potassium stimulation can be assessed. As such, the eSC neurons represent a model cell for testing the selectivity of the agents described.

The selectivity of the ExL-LH_N/A agent for eDRG over eSC neurons is clearly illustrated in Figure 6. The dose curves document the effectiveness of ExL-LH_N/A in an *in vitro* cell culture model by comparing inhibition of neurotransmission in eDRG with eSC neurons.

Materials

Substance P enzyme linked immunosorbent assay kits were from Cayman Chemical Company.

Western blot reagents were obtained from Novex

Monoclonal antibody SMI-81 was from Sternberger Monoclonals Inc.

Methods

Primary cultures of dorsal root ganglion and embryonic spinal cord neurons were established following dissociation of the ganglia dissected from rat embryos (embryological age 12-15 days). For the preparation of

eDRG neurons, the cells were plated into 12 well plates at an initial density of 3 x 10^5 cells/well in a medium containing NGF (100 ng/ml). After one day in culture, fresh medium containing cytosine arabinoside (10 X10^{-6} M) was added to kill non-neuronal cells. After 2-4 days the cytosine arabinoside was removed. After several more days in culture the medium was replaced with fresh medium containing conjugate or LH_N.

For the preparation of eSC neurons, Cells were plated onto poly-D-lysine coated 12 well plates (Costar) at a density of 2x10^6 cells per well (1 ml/well). 'Plating' medium was MEM with Earles Salts (Sigma), containing 5% foetal bovine serum (FBS), 5% heat inactivated horse serum (HS), 0.6% dextrose, 1.5g/l $NaHCO_3$ and 2 mM L-glutamine. Cultures are incubated at 37°C with 10% CO_2. The medium was changed to 'feeding' medium (plating medium minus the FBS with N1 (Sigma) 1/50 supplement) after one day. When glial cells became almost confluent anti-mitotic agents (15 microgrammes /ml 5-fluoro-2'-deoxyuridine (FdU) and 35 microgrammes /ml uridine (U)) were added for a further 2-3 days. Cells were cultured for at least 3 weeks prior to use.

The cells were incubated with these agents for varying times and then tested for their ability to release the neurotransmitters glutamate and substance P (eDRG) or glycine (eSC). After the release assays were performed the cells were lysed and the hydrophobic proteins were extracted by phase partitioning with Triton-X-114 following the method outlined in Boyd, Duggan, Shone and Foster (J. Biol. Chem. **270**, 18216-18218, *1995*).

Substance P release assay

The release of endogenous substance P was effected by collecting cell supernatants after treating the cells for 5 min with either a physiological balanced salt solution

or a balanced salt solution in which the potassium ion concentration had been raised to 100 mM with consequent reduction in the sodium ion concentration to maintain isotonicity. Total substance P was measured after extraction in 2 M acetic acid, 0.1% trifluoroacetic acid and subsequent dehydration. Substance P immunoreactivity was measured using an enzyme immunoassay kit (Cayman Chemical Company).

[^{3}H]Glutamate release assay

The release of glutamate was measured after loading the cells with [^{3}H]glutamine as a radiotracer. The [^{3}H]glutamine is converted to [^{3}H]glutamate in the cell, and it is this [^{3}H]glutamate that is taken up by synaptic vesicles and released upon depolarisation of the neuron. The cells are loaded with the [^{3}H]glutamine (5 X10^{-6} Ci/ml in HEPES-buffered MEM) for 2 h, then washed twice with HEPES-buffered MEM and thrice with balanced salt solution (BSS). Basal release was assessed with a 3 min incubation with BSS. Stimulated release was determined by a 3 min incubation with BSS in which the potassium concentration had been elevated to 80-100 mM with a consequent reduction in the sodium concentration to maintain isotonicity. All manipulations were performed at 37°C. The cells were lysed by the addition of Triton-X-100 (0.1%, v/v). For the basal and stimulated release superfusates the glutamate was separated from the glutamine by ion-exchange chromatography over Dowex-1 resin. The relevant fractions were analysed for ^{3}H content by liquid scintillation counting.

[^{3}H] Glycine release assay

The release of glycine was measured after loading the cells with [^{3}H]glycine as a radiotracer. The [^{3}H]glycine is taken up by synaptic vesicles and released upon depolarisation of the neuron. The cells are loaded with

the [^{3}H]glycine (2 X10^{-6} Ci/ml in HEPES-buffered MEM) for 2 h, then washed once with HEPES-buffered MEM and thrice with balanced salt solution (BSS). Basal release was assessed with a 5 min incubation with BSS. Stimulated release was determined by a 5 min incubation with BSS in which the potassium concentration had been elevated to 56 mM with a consequent reduction in the sodium concentration to maintain isotonicity. All manipulations were performed at 37°C. The cells were lysed by the addition of 2 M acetic acid, 0.1% trifluoroacetic acid. Fractions were analysed for their ^{3}H content by liquid scintillation counting and inhibition of release determined.

Figure 6 illustrates the activity of ExL-LH_N/A on release of neurotransmitter from eDRG and eSC neurons. Both eDRG and eSC cultures were exposed to a range of ExL-LH_N/A concentrations (1 ml volumes) for three days. The percentage inhibition of eDRG substance P (n) and eSC [^{3}H]-glycine (?) release is in comparison to untreated controls. The data shown is representative of =3 determinations. IC_{50} for eDRG was determined to be 3.66±0.92μg/ml. An inhibition of 50% was not obtained for eSC using the concentration range employed.

Western blotting

ExL-LH_N/A was applied to eDRG for 16 hours. After the determination of neurotransmitter release the cells were lysed by the addition of 2 M acetic acid, 0.1% trifluoroacetic acid and subsequently dehydrated. To extract the membrane proteins from these mixtures Triton-X-114 (10%, v/v) was added and incubated at 4°C for 60 min, the insoluble material was removed by centrifugation and the supernatants were then warmed to 37°C for 30 min. The resulting two phases were separated by centrifugation and the upper phase discarded. The proteins in the lower phase were precipitated with chloroform/methanol for analysis by Western blotting.

The extracted protein samples were applied to 4-20% polyacrylamide gradient gels and subjected to electrophoresis prior to transfer to nitrocellulose. Proteolysis of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of BoNT/A, was then detected by probing with an antibody (SMI-81) that recognises both the intact and cleaved forms of SNAP-25 (Figure 2). Proteins blotted onto nitrocellulose were probed with antibody SMI-81. Lanes 1-3, 4-6, 7-9 and 10-12 represent cells treated with medium, 40 microgrammes/ml ExL, 20 microgrammes/ml ExL and 40 microgrammes/ml LH_N/A respectively. Densitometric analysis of these data determined the %SNAP-25 cleavage to be 52.7% and 37.0% for 40 and 20 microgrammes/ml respectively.

Example 5. Activity of SBA-LH_N/A in primary neuronal cultures

Using methodology described in Example 4, the activity of SBA-LH_N/A in primary neuronal cultures was assessed. The selectivity of the SBA-LH_N/A conjugate for eDRG over eSC neurons is illustrated in Figure 7. Both eDRG and eSC cultures were exposed to a range of SBA-LH_N/A concentrations (1 ml volumes) for three days. The percentage inhibition of eDRG substance P (n) and eSC [^{3}H]-glycine (O) release is in comparison to untreated controls. The data is the mean of three determinations $\pm$ SE. The curves shown are representative of two experiments. IC_{50} values for eDRG neurons were determined to be 1.84 and 7.6 microgrammes/ml. It is observed that SBA-LH_N/A exhibits a clear selectivity of the inhibition of neurotransmitter release from eDRG relative to eSC neurons. These data therefore confirm observations described for ExL-LH_N/A above and highlight the properties of galactose-specific lectins.

Example 7. Activity of WGA-LH_N/A in primary neuronal cultures

Using methodology described in Example 4, the activity of WGA-LH_N/A in primary neuronal cultures was assessed. WGA represents an example of a non-galactosyl targeted lectin and therefore serves as an indicator of the properties of conjugate that do not recognise galactosyl moieties. The lack of selectivity of the WGA-LH_N/A conjugate for eDRG over eSC neurons is illustrated in Figure 8. eDRG and eSC neurons were exposed to a range of concentrations of WGA-LH_N/A for 3 days prior to assay of stimulated release of neurotransmitter (substance P and glycine respectively). Each conjugate concentration was assessed in triplicate and results are expressed as percentage inhibition compared to untreated controls. Panels A and B represent dose response curves from one experiment representative of ≥3 for eDRG and eSC neurons respectively. Each point shown is the mean of three determinations ± SE of the mean. IC_{50} data for the effects of WGA-LH_N/A was calculated to be 0.34±0.06 micrograms /ml (eDRG) and 0.06±0.09 micrograms /ml (eSC), indicating the lack of C-fibre selectivity.

Example 8. Activity of ExL-LH_N/A in an electrophysiological model of pain

A dose of 45 micrograms of ExL-LH_N/A in a 10 microlitres volume of vehicle was given by intrathecal injection to rats between lumbar sections L4-L5, 24 hours prior to electrophysiological analysis of neuronal activity. Animals were allowed to recover and movement was not restricted prior to sacrifice and analysis. The results from a group of 3 animals with 10 neurons recorded per animal, show that there was a 73% reduction in the C-fibre responses of the neurones (Figure 9A) although the stimulus threshold is only slightly elevated (Figure 9B). Inhibition of C-fibre responses would lead to a decrease

in the transmission of pain signals and these data are indicative of the analgesic effect of conjugate ExL-LH_N/A. There was also a significant decrease in the A_δ response (Figure 9C). These fibres are also implicated in the transmission of noxious stimuli and this result emphasises the analgesic effect of ExL-LH_N/A. A_β neurons, a cell type that is not involved in transmission of noxious stimuli, were essentially unaltered in their responses to this stimulus (Figure 9D). The lack of affect on the A_β-fibre neurons is indicative of the selectivity of ExL-LH_N/A for the neurons central to the transmission of pain signals.

Example 9. Activity of ExL-LH_N/A in behavioural models of pain

In an accepted *in vivo* model of pain, the mouse hotplate test, ExL-LH_N/A has been demonstrated to exhibit analgesic properties. Figure 10 illustrates the data obtained for ExL-LH_N/A where it is compared to a supramaximal dose of morphine. ExL-LH_N/A was applied intrathecally (30 microgrammes in a 5 microlitre vehicle volume) to each of a group of 10 mice and analgesic response in the hot plate test determined. Data is presented as hot plate latency (seconds) plotted against assay time (P = pre-treatment, 0-5 = hours post application). Onset of ExL-LH_N/A action had apparently reached a plateau at 1 hour that remained constant for at least 5 hours. The level of analgesia is similar to a supramaximal dose (50 microgrammes, 20X mouse EC_{50}) of morphine in this test, but is of much longer duration. This level of morphine achieves a maximal effect at 1 hour and then returns to control levels over a period of 5 hours. These data represent a clear indication of the analgesic potential of agents such as ExL-LH_N/A.

Materials

Adult outbred mice (MF1) of either sex, weight range 20 to 30g.

Methods

Test material is injected into the intrathecal space of anaesthetised mice using a 30 gauge disposable needle attached to a 50 microlitre Hamilton syringe. The site of injection was normally chosen to be between lumbar vertebrae 5 and 6. The needle is inserted into the tissue to one side of the vertebrae so that it slips into the groove between the spinous and transverse processes. The needle is then moved carefully forward to the intervertebral space. 5 microlitres of test material is then injected into the intrathecal space and the needle withdrawn. The skin incision is then closed with a single wound clip and the animal placed in a box to allow recovery.

Claims

1. An agent for the treatment of pain that comprises a galactose-binding lectin linked to a derivative of a clostridial neurotoxin, in which the derivative of the clostridial neurotoxin comprises the L-chain, or a fragment thereof, which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.

2. An agent according to Claim 1 in which the membrane translocation domain is derived from the heavy chain of a clostridial toxin.

3. An agent according to Claim 1 in which the membrane translocation domain is derived from a non-clostridial source.

4. An agent according to any preceding Claim in which the lectin binds to oligosaccharides that contain terminal β-D-galactosyl residues

5. An agent according to any preceding Claim in which the lectin binds to oligosaccharides that contain terminal α-D-galactosyl residues

6. An agent according to any preceding Claim in which the lectin binds to oligosaccharides that contain N-acetylgalactosamine

7. An agent according to any previous Claim in which the lectin is derived from a species of plant.

8. An agent according to the previous Claim in which the lectin is derived from a species of the genus *Erythrina*.

9. An agent according to Claim 8 in which the lectin is derived from *E. cristagalli*.

10. An agent according to Claim 8 in which the lectin is derived from *E. corallodendron*.

11. An agent according to Claims 7 in which the lectin is obtained from *Glycine max*.

12. An agent according to Claims 7 in which the lectin is obtained from *Arachis hypogaea*.

13. An agent according to Claims 7 in which the lectin is obtained from *Bandeirea simplicifolia*.

14. An agent according to Claim 1-6 in which the lectin is of mammalian origin.

15. An agent according to Claim 1-6 in which the lectin is obtained from bacteria.

16. An agent according to Claim 15 in which the lectin is obtained from *Pseudomonas aeruginosa*.

17. An agent according to any preceding Claim in which the lectin has been produced using recombinant technology.

18. An agent according to any preceding Claim in which the lectin has been enzymatically modified.

19. An agent according to any preceding Claim in which the lectin has been chemically modified.

20. An agent according to any preceding Claim which comprises the lectin coupled to a clostridial neurotoxin in which the H_c domain of the H-chain is removed or modified.

21. An agent according to any preceding Claim in which the H-chain is modified by chemical derivatisation to reduce or remove its native binding affinity for motor neurons.

22. An agent according to any of Claims 1-20 in which the H-chain is modified by mutation to reduce or remove its native binding affinity for motor neurons.

23. An agent according to any of Claims 1-20 in which the H-chain is modified by proteolysis.

24. An agent according to Claim 20 in which the H_c domain is completely removed leaving only the H_N-fragment of a clostridial neurotoxin.

25. An agent according to any preceding Claim in which the clostridial neurotoxin component is obtained from botulinum neurotoxin.

26. An agent according to any preceding Claim in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type A.

27. An agent according to Claims 1-25 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type B.

28. An agent according to any of Claims 1-25 which is formed by the coupling of a galactose-binding lectin to the LH_N fragment of botulinum neurotoxin type A.

29. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Erythrina cristagalli* to the LH_N fragment of botulinum neurotoxin type A.

30. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Erythrina corallodendron* to the LH_N fragment of botulinum neurotoxin type A.

31. An agent according to Claim 28 which is formed by the coupling of the galactose-binding lectin from *Glycine max* to the LH_N fragment of botulinum neurotoxin type A.

32. An agent according to any preceding Claim in which the H-chain is obtained from a different clostridial neurotoxin than that from which the L-chain is obtained.

33. An agent according to Claim 32 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from botulinum neurotoxin type B.

34. An agent according to Claim 32 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from tetanus neurotoxin.

35. An agent according to Claims 33 and 34 in which the H-chain component is the H_N fragment of botulinum neurotoxin type A.

36. An agent according to any preceding Claim in which the L-chain or L-chain fragment is linked to the H-chain by a direct covalent linkage.

37. An agent according to any of Claims 1-35 in which the L-chain or L-chain fragment is linked to the H-chain by a covalent linkage which includes one or more spacer regions.

38. An agent according to any preceding Claim in which the clostridial neurotoxin derivative incorporates polypeptides produced by recombinant technology.

39. An agent according to any preceding Claim in which the lectin is linked to the clostridial neurotoxin-derived component by a direct covalent linkage.

40. An agent according to any of Claims 1-38 in which the lectin is linked to the clostridial neurotoxin-derived component by a covalent linkage which includes one or more spacer regions.

41. An agent according to any preceding Claim in which the lectin and clostridial neurotoxin components are produced as a recombinant fusion protein.

42. An agent according to any preceding Claim in which the lectin protein has been modified from its native polypeptide sequence whilst retaining an ability for the protein to bind to oligosaccharide structures, in which the terminal residue is derived from galactose or N-acetylgalactosamine.

43. An agent according to Claim 42 in which the protein modification results from modification of the nucleic acid coding for the lectin protein from its native sequence.

44. An agent according to any preceding Claim which prevents the release of a neurotransmitter or neuromodulator from a primary sensory afferent.

45. An agent according to any preceding Claim which inhibits the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent.

46. A method for obtaining an agent according to any preceding Claim which comprises the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin, in which the derivative of the clostridial neurotoxin comprises the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.

47. A method for obtaining an agent according to any of Claims 1-58 which comprises the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin with the inclusion of one or more spacer regions, in which the derivative of the clostridial neurotoxin comprises the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to a molecule or domain with membrane translocating activity.

48. An method according to Claim 47 in which the membrane translocation domain is derived from the heavy chain of a clostridial toxin.

49. An method according to Claim 47 in which the membrane translocation domain is derived from a non-clostridial source.

50. A method for obtaining an agent according to any of Claims 1-45 which comprises constructing a genetic construct which codes for the agent, incorporating said construct into a host organism and expressing the construct to produce the agent.

51. A method of controlling the release of a neurotransmitter or neuromodulator from a primary sensory afferent by applying the agent of any one of Claims 1-45.

52. A method of controlling the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent by applying the agent of any one of Claims 1-45.

53. A method of controlling the transmission of sensory information from a primary sensory afferent to a projection neuron by applying the agent of any one of Claims 1-45.

54. A method of controlling the transmission of sensory information from a primary nociceptive afferent to a projection neuron by applying the agent of any one of Claims 1-45.

55. A method of controlling the sensation of pain by applying the agent of any one of Claims 1-45.

56. Use of the agent according to any one of Claims 1-45 or a pharmaceutically acceptable salt thereof as a medicament for the alleviation of pain.

57. Use of the agent according to any one of Claims 1-45 or a pharmaceutically acceptable salt thereof as a medicament for the prevention of pain.

58. Use of the agent according to any one of Claims 1-45 in the manufacture of a medicament for the alleviation of pain.

59. Use of the agent according to any one of Claims 1-45 in the manufacture of a medicament for the prevention of pain.

60. A method of alleviating pain which comprises administering an effective dose of the agent according to any one of Claims 1-45.

61. A method of preventing pain which comprises administering an effective dose of the agent according to any one of Claims 1-45.

Figure 1

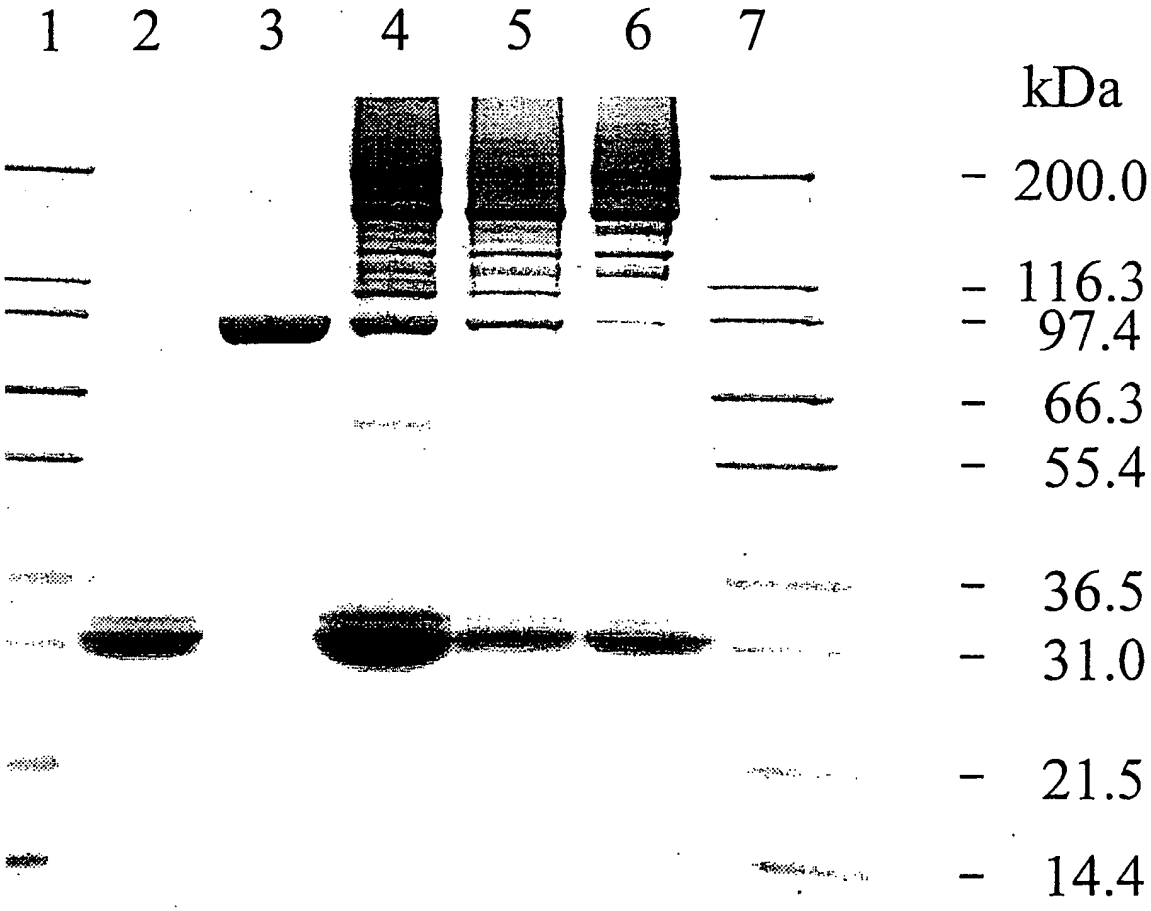


Figure 2

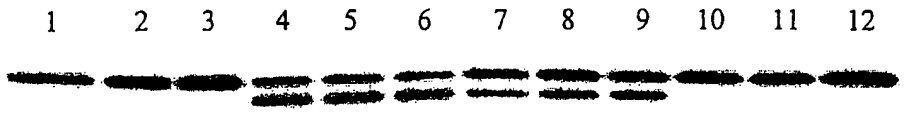


Figure 3

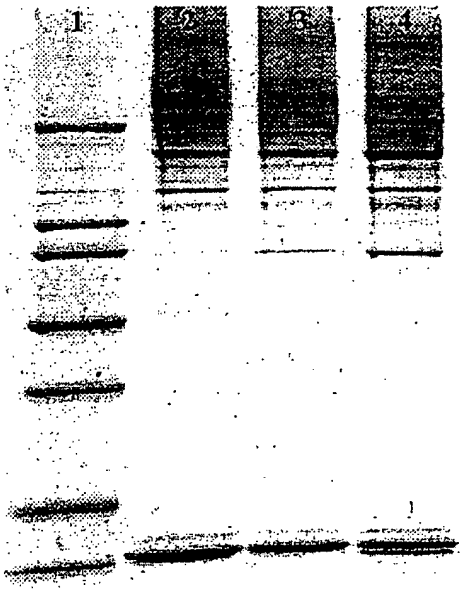


Figure 4

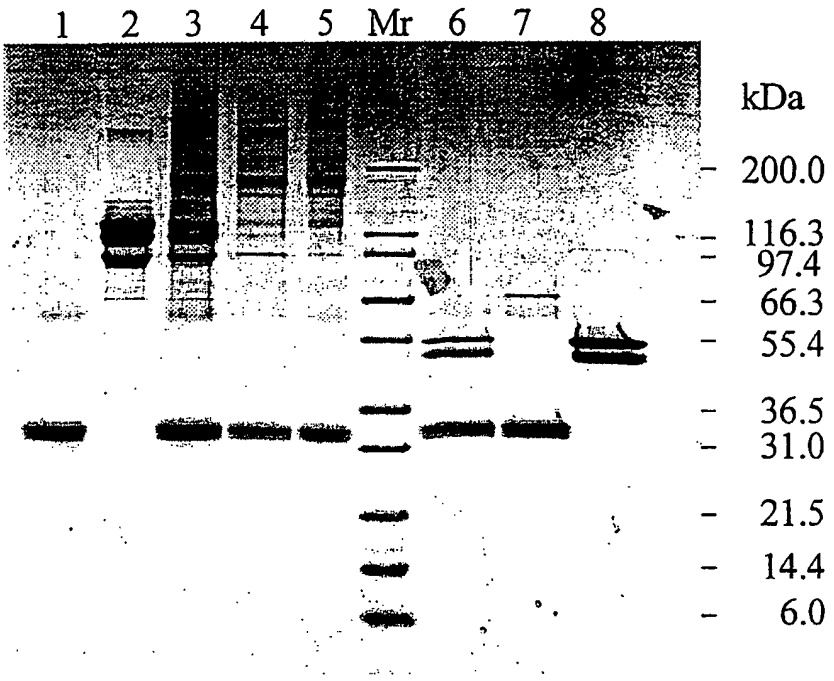


Figure 5

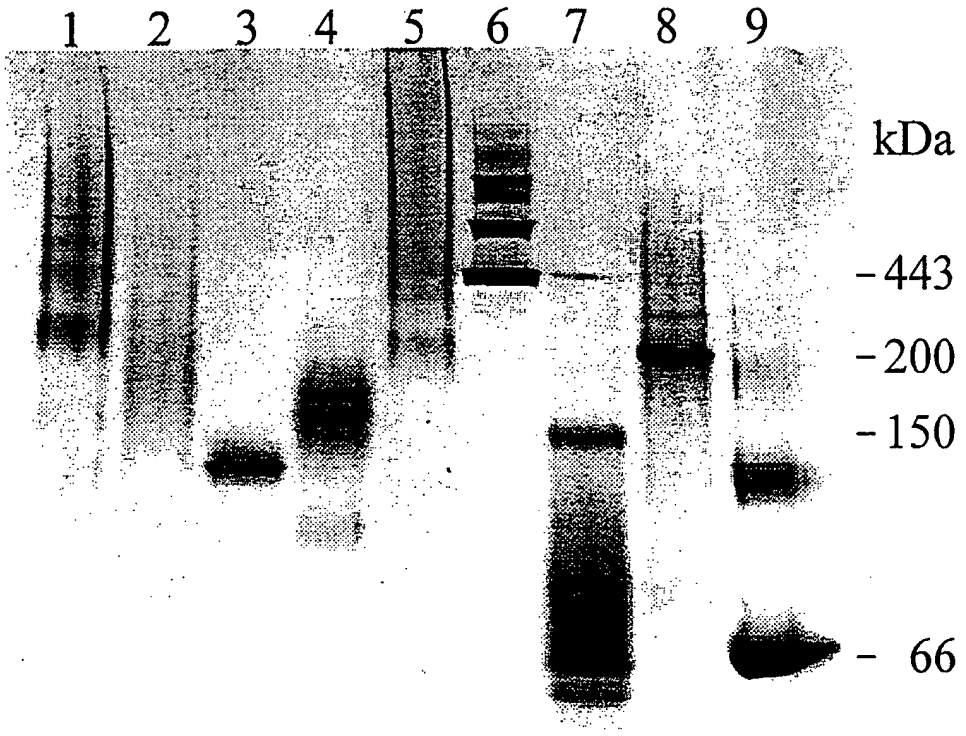


Figure 6

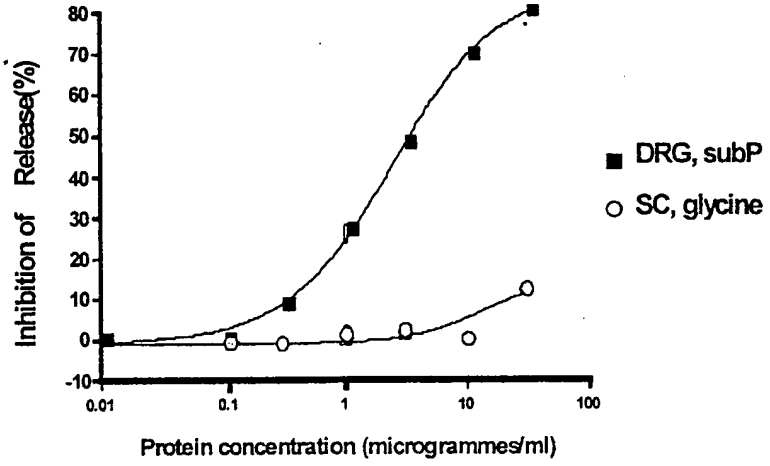

Inhibition of Release(%)
80
70
60
50
40
30
20
10
0
-10
0.01
0.1
1
10
100
Protein concentration (microgrammes/ml)
DRG, subP
SC, glycine

Figure 7

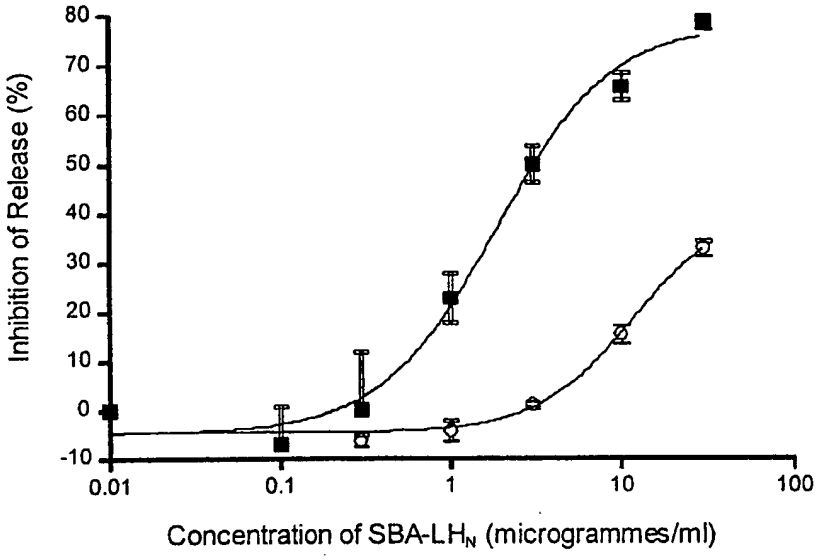
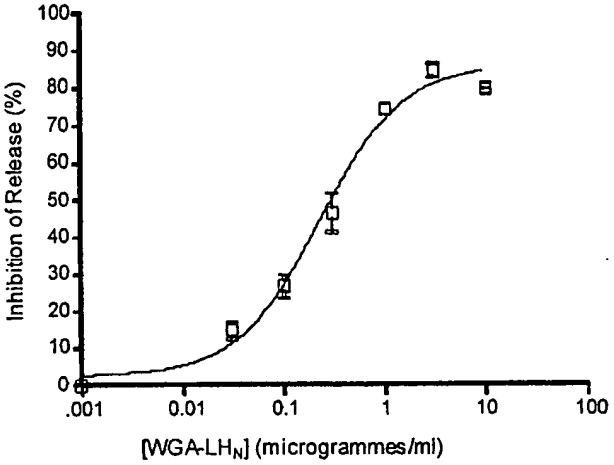

80
70
60
50
40
30
20
10
0
-10
Inhibition of Release (%)
0.01
0.1
1
10
100
Concentration of SBA-LH$_N$ (microgrammes/ml)

Figure 8

Panel A: eDRG



Panel B: eSC neurons

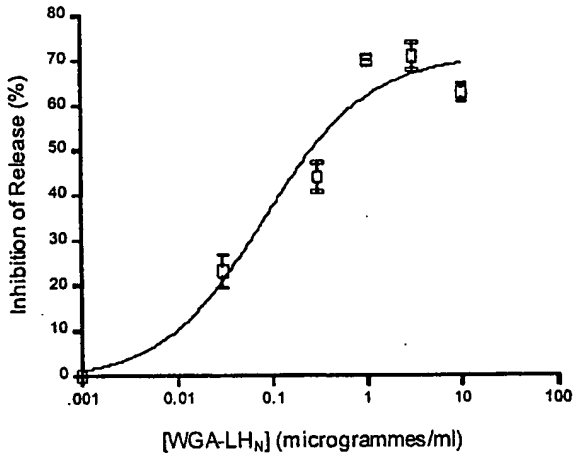
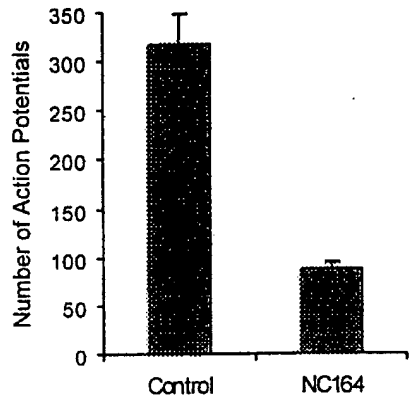
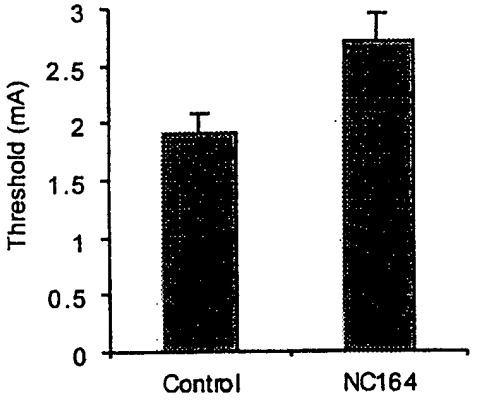


Figure 9

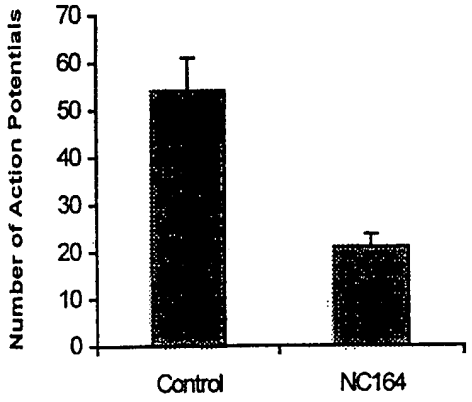
A.


Number of Action Potentials
350
300
250
200
150
100
50
0
Control
NC164

B.


Threshold (mA)
3
2.5
2
1.5
1
0.5
0
Control
NC164

C.


Number of Action Potentials
70
60
50
40
30
20
10
0
Control
NC164

D.

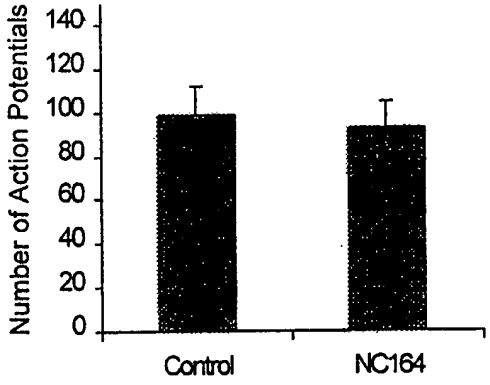
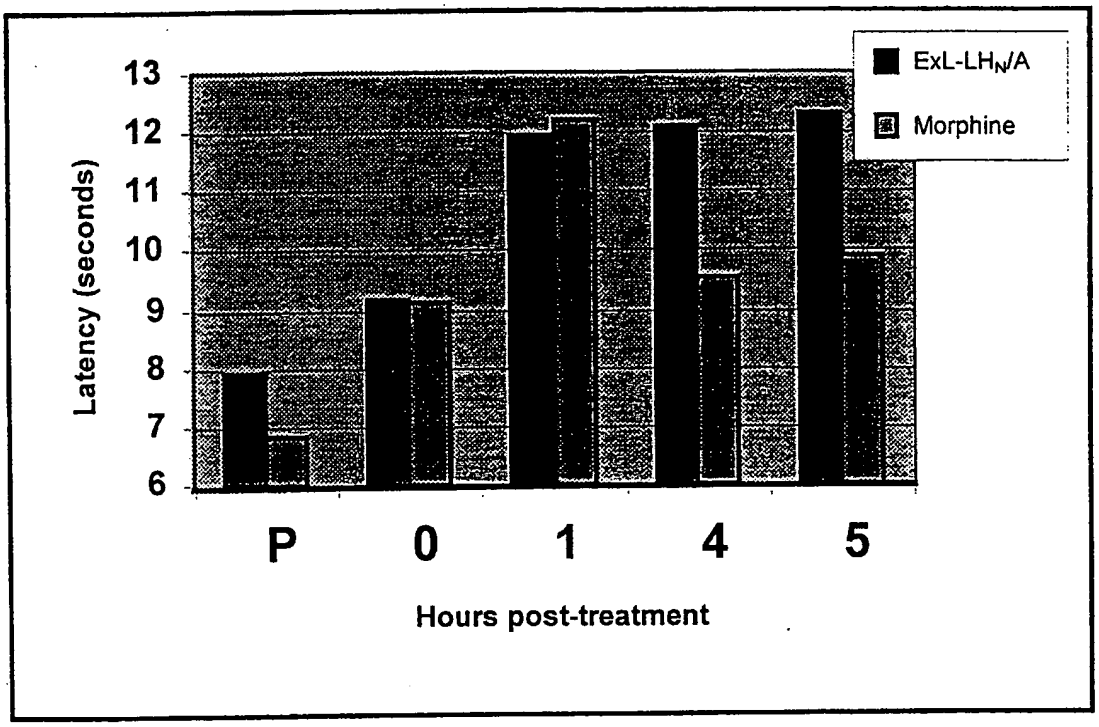

Number of Action Potentials
140
120
100
80
60
40
20
0
Control
NC164

Figure 10


ExL-LH$_N$/A
Morphine
Latency (seconds)
13
12
11
10
9
8
7
6
P
0
1
4
5
Hours post-treatment

INTERNATIONAL SEARCH REPORT

Int. .tional Application No
PCT/GB 98/03001

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 33273 A (SPEYWOOD LAB LIMITED ;MICROBIOLOGICAL RES AUTHORITY (GB); FOSTER K) 24 October 1996 cited in the application	1-61
Y	see page 12, line 1 - page 13, line 8; claims see page 13, line 9 - line 13	1-61
P,X	WO 98 07864 A (FOSTER KEITH ALAN ;MICROBIOLOGICAL RES AUTHORITY (GB); QUINN CONRA) 26 February 1998 see claims	1
A	WO 94 28923 A (ALLERGAN INC) 22 December 1994 see claims	

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 January 1999	09/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Berte, M

2

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/03001

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 18790 A (UNIV MONTANA RES DEV INST ;PASCUAL DAVID (US); BOND CLIFFORD (US);) 29 May 1997 see claims 1,4,5,9,29,30 ---	1-61
A	WO 95 32738 A (ALLERGAN INC ;DOLLY JAMES OLIVER (GB); AOKI KEI ROGER (US); WHEELE) 7 December 1995 ---	
Y	STREIT, WOLFGANG J. ET AL: "Histochemical localization of galactose-containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of the rat" J. HISTOCHEM. CYTOCHEM. (1985), 33(10), 1042-52 CODEN: JHCYAS;ISSN: 0022-1554, 1985, XP002091075 see abstract -----	1-61

2

INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/GB 98/03001

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 51-55,60-61
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/GB 98/03001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9633273 A	24-10-1996	AU 5339896 A	07-11-1996
		BG 101984 A	31-07-1998
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		AU 2622295 A	21-12-1995
		CA 2191754 A	07-12-1995
		EP 0760681 A	12-03-1997
		JP 10500988 T	27-01-1998

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/31, C07K 19/00, 14/33, C12N 9/52, C07K 14/48, 14/475, 14/52, 14/575, 16/18, C12N 15/62, A61K 38/16

A1

(11) International Publication Number: **WO 96/33273**

(43) International Publication Date: 24 October 1996 (24.10.96)

(21) International Application Number: PCT/GB96/00916

(22) International Filing Date: 16 April 1996 (16.04.96)

(30) Priority Data:
9508204.6 21 April 1995 (21.04.95) GB

(71) Applicants *(for all designated States except US)*: THE SPEYWOOD LABORATORY LIMITED [GB/GB]; 14 Kensington Square, London W8 5HH (GB). MICROBIOLOGICAL RESEARCH AUTHORITY [GB/GB]; Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire SP4 0JG (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FOSTER, Keith, Alan [GB/GB]; The Croft, Southampton Road, Whaddon, Salisbury, Wiltshire SP5 3DX (GB). DUGGAN, Michael, John [GB/GB]; 27A Moreton Place, Pimlico, London SW1V 2NL (GB). SHONE, Clifford, Charles [GB/GB]; 44 Oakwood Grove, Alderbury, Wiltshire SP5 3BN (GB).

(74) Agent: DAVIES, Jonathan, Mark; Reddie & Grose, 16 Theobalds Road, London WC1X 8PL (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: BOTULINUM TOXIN DERIVATIVES ABLE TO MODIFY PERIPHERAL SENSORY AFFERENT FUNCTIONS

(57) Abstract

The invention relates to an agent specific for peripheral sensory afferents. The agent may inhibit the transmission of signals between a primary sensory afferent and a projection neuron by controlling the release of at least one neurotransmitter or neuromodulator from the primary sensory afferent. The agent may be used in or as a pharmaceutical for the treatment of pain, particularly chronic pain.

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Botulinum toxin derivatives able to modify peripheral sensory afferent functions

Technical Field

This invention relates to a novel agent that is able to modify peripheral afferent function. The agent may inhibit neurotransmitter release from discrete populations of neurons, and thereby reduce, or preferably prevent, the transmission of afferent pain signals from peripheral to central pain fibres. The agent may be used in or as a pharmaceutical for the treatment of pain, particularly chronic pain.

Background

The sense of touch has traditionally been regarded as one of the five classical senses, but in reality it is highly complex, transducing a number of different sensations. These sensations are detected in the periphery by a variety of specialised nerve endings and associated structures. Some of these are specific for mechanical stimuli of various sorts such as touch, pressure, vibration, and the deformation of hairs or whiskers. Another class of nerves is able to detect temperatures, with different fibres being activated by heat and cold. A further population of nerve endings is not normally excited by mild stimuli, but by strong stimuli only. Sensory nerves of this category often respond to more than one stimulus, and are known as high-threshold polymodal fibres. They may be used to sense potentially damaging situations or objects. The polymodal fibres also transduce chemical signals such as the "burning" sensation evoked by acid. Thus, the sense of touch can transmit a very detailed description of objects and serve to both inform and warn of events.

The transduction of sensory signals from the periphery to sensation itself is achieved by a multi-neuronal pathway and the information processing centres of the brain. The first nerve cells of the pathway involved in the transmission of sensory stimuli are called primary sensory afferents. The cell bodies for the primary sensory afferents from the head and some of the internal organs reside in various of the ganglia associated with the cranial nerves, particularly the trigeminal nuclei and the nucleus of the solitary tract. The cell bodies for the primary sensory afferents for the remainder of the body lie in the dorsal root ganglia of the spinal column. The primary sensory afferents and their processes have been classified histologically; the cell bodies fall into two classes: A-type are large (60-120 μm in diameter) while B-type are smaller (14-30 μm) and more numerous. Similarly the processes fall into two categories: C-fibres lack the myelin sheath that A-fibres possess. A-fibres can be further sub-divided into Aβ-fibres, that are large diameter with well developed myelin, and Aδ-fibres, that are thinner with less well developed myelin. It is generally believed that Aβ-fibres arise from A-type cell bodies and that Aδ- and C-fibres arise from B-type cell bodies. These classifications can be further extended and subdivided by studying the selective expression of a range of molecular markers.

Functional analyses indicate that under normal circumstances Aβ-fibres transmit the senses of touch and moderate temperature discrimination, whereas the C-fibres are mainly equivalent to the polymodal high-threshold fibres mentioned above. The role of Aδ-fibres is less clear as they seem to have a variety of responsive modes, with both high and low thresholds.

After the activation of the primary sensory afferents the next step in the transduction of sensory signals is the activation of the projection neurons, which carry the signal to higher parts of the central nervous system such as the thalamic nuclei. The cell bodies of these neurons (other than those related to the

cranial nerves) are located in the dorsal horn of the spinal cord. This is also where the synapses between the primary afferents and the projection neurons are located. The dorsal horn is organised into a series of laminae that are stacked, with lamina I being most dorsal followed by lamina II, etc. The different classes of primary afferents make synapses in different laminae. For cutaneous primary afferents, C-fibres make synapses in laminae I and II, Aδ-fibres in laminae I, II, and V, and Aβ-fibres in laminae III, IV, and V. Deeper laminae (V-VII, X) are thought to be involved in the sensory pathways arriving from deeper tissues such as muscles and the viscera.

The predominant neurotransmitter at the synapses between primary afferents and projection neurons is glutamate, although importantly the C-fibres contain several neuropeptides such as substance P and calcitonin-gene related peptide (CGRP). A-fibres may also express neuropeptides such as neuropeptide Y under some circumstances.

The efficiency of transmission of these synapses can be altered via descending pathways and by local interneurons in the spinal cord. These modulatory neurons release a number of mediators that are either inhibitory (e.g. opioid peptides, glycine) or excitatory (e.g. nitric oxide, cholecystokinin), to provide a mechanism for enhancing or reducing awareness of sensations.

A category of sensation that requires such physiological modulation is pain. Pain is a sensation that can warn of injury or illness, and as such is essential in everyday life. There are times, however, when there is a need to be able to ignore it, and physiologically this is a function of, for example, the opioid peptides. Unfortunately, despite these physiological mechanisms, pain can continue to be experienced during illnesses or after injuries long after its utility has passed. In these circumstances pain becomes a symptom of disease that would be better alleviated.

Clinically, pain can be divided into three categories: (1) Acute pain, usually arising from injury or surgery that is expected to disappear when that injury is healed. (2) Chronic pain arising from malignant disease; the majority of people with metastatic cancer have moderate to severe pain and this is resolved either by successful treatment of the disease or by the death of the patient. (3) Chronic pain not caused by malignant disease; this is a heterogeneous complaint, caused by a variety of illnesses, including arthritis and peripheral neuropathies, that are usually not life-threatening but which may last for decades with increasing levels of pain.

The physiology of pain that results from tissue damage is better understood than that which is caused by central nervous system defects. Under normal circumstances the sensations that lead to pain are first transduced by the Aδ- and C-fibres that carry high threshold signals. Thus the synapses in laminae I and II are involved in the transmission of the pain signals, using glutamate and the peptides released by C-fibres to produce activation of the appropriate projection neurons. There is, however, evidence that in some chronic pain states other A-fibres (including Aβ-fibres) can carry pain signals, and thus act as primary nociceptive afferents, for example in the hyperalgesia and allodynia associated with neuropathic pain. These changes have been associated with the expression of peptides such as neuropeptide Y in A fibres. During various chronic pain conditions the synapses of the various sensory afferents with projection neurons may be modified in several ways: there may be changes in morphology leading to an increase in the number of synapses, the levels and ratios of the different peptides may change, and the sensitivity of the projection neuron may change.

Given the enormity of the clinical problem presented by pain, considerable effort has been expended in finding methods for its alleviation. The most commonly used pharmaceuticals for the alleviation of pain fall into two

categories: (1) Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and ibuprofen; (2) Opioids, including morphine.

NSAIDs have their main analgesic action at the periphery by inhibiting the production of prostaglandins by damaged tissues. Prostaglandins have been shown to be peripheral mediators of pain and inflammation and a reduction in their concentration provides relief to patients. This is especially the case in mild arthritic disease, where inflammation is a major cause of pain. It has been suggested that prostaglandins are involved in the mediation of pain in the spinal cord and the brain; this may explain why NSAIDs have analgesic effects in some pain states that do not involve inflammation or peripheral tissue damage. As prostaglandins, however, are only one of several mediators of pain NSAIDs alone are only effective in reducing some types of mild pain to acceptable levels. They are regarded as having a ceiling of activity above which increasing doses do not give increasing pain relief. Furthermore they have side effects that limit their usefulness in chronic complaints. The use of NSAIDs is associated with irritation of the gastro-intestinal tract and prolonged use may lead to the development of extensive ulceration of the gut. This is particularly true in elderly patients who form the largest cohort of patients with, for example, arthritis.

Opioids act at the level of the spinal cord to inhibit the efficiency of neurotransmission between the primary nociceptive fibres (principally C-fibres) and the projection neurons. They achieve this by causing a prolonged hyperpolarization of both elements of these synapses. The use of opioids is effective in alleviating most types of acute pain and chronic malignant pain. There are, however, a number of chronic malignant pain conditions which are partly or completely refractory to opioid analgesia, particularly those which involve nerve compression, e.g. by tumour formation. Unfortunately opioids also have unwanted systemic side-effects including: (1) depression of the

respiratory system at the level of the respiratory centres in the brain stem; (2) the induction of constipation by a variety of effects on the smooth musculature of the gastro-intestinal tract; and (3) psychoactive effects including sedation and the induction of euphoria. These side effects occur at doses similar to those that produce analgesia and therefore limit the doses that can be given to patients.

Delivery of opioids at the spinal level can reduce the side-effect profile, but requires either frequently repeated spinal injections or fitting of a catheter, both of which carry increased risk to the patient. Fitting of a catheter requires that the patient is essentially confined to bed thus further restricting their quality of life.

The use of opioids for the treatment of some other types of chronic pain is generally ineffective or undesirable. Examples include the pain associated with rheumatoid arthritis and neuromas that develop after nerve injury. The undesirable nature of opioid treatment in these patients is related not only to side-effects already mentioned and the probable duration of the disease but also to the fourth major side-effect of the opioids: dependence. Opioids such as morphine and heroin are well-known drugs of abuse that lead to physical dependence, this last side-effect involves the development of tolerance: the dose of a drug required to produce the same analgesic effect increases with time. This may lead to a condition in which the doses required to alleviate the pain are life-threatening due to the first three side-effects.

Although NSAIDs and opioids have utility in the treatment of pain there is general agreement that they are often not appropriate for the adequate treatment of pain, particularly chronic and severe pains.

Other treatments are also used, particularly for the treatment of chronic severe pain including surgical lesions of the pain pathways at several levels from

peripheral nerves through dorsal root section and cordotomy to pituitary destruction. These are, however, mostly severe operations that are all associated with significant risk to the patient.

It can be seen, therefore, that there remains a significant need for the development of new classes of pharmaceuticals for the treatment of pain of many types. The desired properties of such new therapies can be briefly expressed as follows: (1) the ability to provide significant relief of pain including severe pain; (2) the lack of systemic side effects that significantly impair the patient's quality of life; (3) long-lasting actions that do not require frequent injections or long-term catheterisation of patients; (4) provision of agents that do not lead to tolerance and associated dependence.

Statement of Invention

The present invention relates to an agent which can reduce and preferably prevent the transmission of pain signals from the periphery to the central nervous system, thereby alleviating the sensation of pain. Specifically, the invention can provide an agent which can reduce and preferably prevent the transmission of pain signals from nociceptive afferents to projection neurons. More specifically, the invention can provide an agent which can inhibit the exocytosis of at least one neurotransmitter or neuromodulator substance from at least one category of nociceptive afferents.

In a first aspect of the invention, there is provided an agent which can be administered systemically, and can specifically target defined populations of nociceptive afferents to inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nerves.

In a second aspect of the invention, there is provided an agent which can be locally administered at the periphery, and which is able to inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents transmitting the pain signal from the periphery.

In a third aspect of the invention, an agent is provided which can be administered into the spinal cord, and which can inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents terminating in that region of the spinal cord.

In a fourth aspect of the invention, there is provided an agent which can specifically target defined populations of afferent neurons, so that the effect of the agent is limited to that cell type.

In a fifth aspect of the invention, there is provided a method of treatment of pain which comprises administering an effective dose of the agent according to the invention.

In a sixth aspect of the invention, the agent can be expressed recombinantly as a fusion protein which includes the required components of the agent.

Definitions

Without wishing to be limited by the definitions set down, it is intended in this description that the following terms have the following meanings:

Light chain means the smaller of the two polypeptide chains which form clostridial neurotoxins; it has a molecular mass of approximately 50 kDa and is commonly referred to as L-chain or simply L.

Heavy chain means the larger of the two polypeptide chains which form clostridial neurotoxins; it has a molecular mass of approximately 100 kDa and is commonly referred to as H-chain or simply H.

H_C fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the carboxy-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. It contains the domain of the natural toxin involved in binding to motor neurons.

H_N fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact in the H-chain. It contains a domain involved in the translocation of the L-chain across endosomal membranes.

LH_N means a fragment derived from a clostridial neurotoxin that contains the L-chain, or a functional fragment thereof coupled to the H_N fragment. It is commonly derived from the intact neurotoxin by proteolysis.

Targeting Moiety (TM) means any chemical structure of an agent which functionally interacts with a binding site causing a physical association between the agent and the surface of a primary sensory afferent.

Binding site (BS) means a structure on the surface of a cell with which exogenous molecules are able to interact in such a way as to bring about a physical association with the cell.

Primary sensory afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system.

Primary nociceptive afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system, where that information can result in a sensation of pain.

Brief Description of the Drawings

Figure 1 shows a Coomassie stain of an SDS-PAGE analysis of the fractions from size-exclusion chromatography of the products of the coupling reaction between derivatized Nerve Growth Factor (NGF) and derivatized LH_N from BoNT/A.

Figure 2 shows a Coomassie stain of an SDS-PAGE analysis of the conjugate of NGF and LH_N under reducing and non-reducing conditions.

Figure 3 shows a Western blot of extracts from PC12 cells treated with the conjugate of NGF and LH_N, probed with an antibody that recognises the product of the proteolysis of SNAP-25 by the L-chain of BoNT/A.

Figure 4 shows a Western blot of extracts from rat dorsal root ganglion neurons treated with the conjugate of NGF and LH_N, probed with an antibody that recognises the product of the proteolysis of SNAP-25 by the L-chain of BoNT/A.

Detailed Description of the Invention

It can be seen that, an agent for reducing or preventing the transmission of pain signals from peripheral, nociceptive afferent neurons to projection neurons has many potential applications in the reduction of the sensation of pain, particularly of severe, chronic pain.

According to the invention, there is provided an agent which can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents.

The agent has a number of discrete functions:

1) It binds to a surface structure (the Binding Site [BS]) which is characteristic of, and has a degree of specificity for, nociceptive afferent neurons.

2) It enters the neuron. The entry of molecules into a cell can occur by a process of endocytosis. Only certain cell surface BSs undergo endocytosis, and preferably the BS to which the agent binds is one of these. In one aspect of this invention, the BS is present on the peripheral, sensory fibres of the nociceptive afferent neuron and, following internalization, undergoes retrograde transport to the cell body and central processes of the neuron, in such a manner that the agent is also delivered to these regions of the neuron. In another aspect of this invention, the BS to which the agent binds is present on the central processes or cell body of the nociceptive afferent neuron.

3) The agent enters the cytosol.

4) The agent modifies components of the exocytotic machinery present in the synaptic terminals of the central processes of those neurons, such that the release of at least one neurotransmitter or neuromodulator from the synaptic terminal is reduced or preferably prevented.

Surprisingly, an agent of the present invention can be produced by modifying a clostridial neurotoxin or fragment thereof.

The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight different classes of the neurotoxins known: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesized by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), which has a molecular mass of approximately 100 kDa, and the light chain (L), which has a molecular mass of approximately 50 kDa. The clostridial neurotoxins bind to an acceptor site on the cell membrane of the motor neuron at the neuromuscular junction and are internalised by an endocytotic mechanism. The internalised clostridial neurotoxins possess a highly specific zinc-dependent endopeptidase activity that hydrolyses a specific peptide bond in at least one of three proteins, synaptobrevin, syntaxin or SNAP-25, which are crucial components of the neurosecretory machinery, and this activity of the clostridial toxins results in a prolonged muscular paralysis. The zinc-dependent endopeptidase activity of clostridial neurotoxins is found to reside in the L-chain. The clostridial neurotoxins are highly selective for motorneurons due to the specific nature of the acceptor site on those neurons. The specific neuromuscular junction binding activity of clostridial neurotoxins is known to reside in the carboxy-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as H_C.

Surprisingly, by covalently linking a clostridial neurotoxin, or a hybrid of two clostridial neurotoxins, in which the H_c region of the H-chain has been removed or modified, to a new molecule or moiety, the Targeting Moiety (TM), that binds to a BS on the surface of sensory neurons, a novel agent capable of inhibiting the release of at least one neurotransmitter or neuromodulator from

nociceptive afferents is produced. A further surprising aspect of the present invention is that if the L-chain of a clostridial neurotoxin, or a fragment of the L-chain containing the endopeptidase activity, is covalently linked to a TM which can also effect internalisation of the L-chain, or fragment thereof, into the cytoplasm of a sensory neuron, this also produces a novel agent capable of inhibiting the release of at least one neurotransmitter or neuromodulator. The covalent linkages used to couple the component parts of the agent may include appropriate spacer regions.

The TM provides specificity for the BS on the nociceptive afferent neuron. The TM component of the agent can comprise one of many cell binding molecules, including, but not limited to, antibodies, monoclonal antibodies, antibody fragments (Fab, $F(ab)'_2$, Fv, ScFv, etc.), lectins and ligands to the receptors for hormones, cytokines, growth factors or neuropeptides. A list of possible TMs is given in Table 1, this list is illustrative and is not intended to be limiting to the scope of TMs which could fulfil the requirements of this invention. In one embodiment of the invention the TM binds to a BS which undergoes retrograde transport.

It is known in the art that the H_C portion of the neurotoxin molecule can be removed from the other portion of the heavy chain, known as H_N, such that the H_N fragment remains disulphide linked to the light chain (L-chain) of the neurotoxin molecule to provide a fragment known as LH_N. Thus, in one embodiment of the present invention the LH_N fragment of a clostridial neurotoxin is covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H_C domain of a clostridial neurotoxin is mutated or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the

neuromuscular junction. This modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the heavy chain of a clostridial neurotoxin, in which the H_C domain is mutated or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction is combined with the L-chain of a different clostridial neurotoxin. The hybrid, modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H_N portion of a clostridial neurotoxin is combined with the L-chain of a different clostridial neurotoxin. The hybrid LH_N is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is linked, using linkages which may include one or more spacer regions, to a TM which can also effect the internalization of the light chain, or fragment thereof containing endopeptidase activity, into the cytoplasm of the cell.

In another embodiment of the invention the agent is expressed recombinantly as a fusion protein which includes an appropriate fragment of a Targeting Moiety in addition to any desired spacer domains. The recombinantly expressed agent may be derived wholly from the gene encoding one serotype of neurotoxin or be a chimaera derived from the genes encoding two different serotypes.

In another embodiment of the invention the required LH_N, which may be a hybrid of an L and H_N from different clostridial toxin types, is expressed recombinantly as a fusion protein with the TM, and may also include one or more spacer regions.

In another embodiment of the invention the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is expressed recombinantly as a fusion protein with a TM which can also affect the internalization of the light chain, or fragment thereof containing the endopeptidase activity, into the cytoplasm of the cell. The expressed fusion protein may also include one or more spacer regions.

The basis of this disclosure is the creation of novel agents with very specific and defined activities against a limited and defined class of neurons (primary sensory afferents), and as such the agents may be considered to represent a form of neurotoxin. The therapeutic use of native botulinum neurotoxins is well known in the prior art. The mode of action of the botulinum neurotoxins, as described in the prior art, however, is by a mechanism, inhibition of acetylcholine secretion, and against a category of target neurons, efferent motorneurons, clearly distinct from the agents described in this disclosure. The prior art does not teach either the activity or the chemical structure of the agents disclosed. Thus, although, as discussed in this application, the prior art teaches much about the native clostridial neurotoxins, native unmodified clostridial neurotoxins are not the subject of this disclosure. The agent of this invention requires modification of the clostridial neurotoxins such that the targeting property taught in the prior art is removed. The modified neurotoxin is then coupled to a new targeting function (the TM), to give a novel agent with new biological properties distinct from those of the native clostridial neurotoxins and not taught in the prior art. It is this new agent with novel properties that is the subject of this disclosure.

Exploitation in Industry

The agent described in this invention can be used in vivo, either directly or as a pharmaceutically acceptable salt, for treatment of pain.

For example, an agent according to the invention can be used systemically for the treatment of severe chronic pain. A specific example of this is the use in treatment of clinical pain associated with rheumatoid arthritis affecting multiple joints.

In another example, an agent according to the invention can be locally applied for the treatment of pain. A specific example of this is treatment by local injection into a joint affected by inflammatory pain.

In further example an agent according to the invention can be administered by spinal injection (epidural or intrathecal) at the level of the spinal segment involved in the innervation of an affected organ for the treatment of pain. This is, for example, applicable in the treatment of deep tissue pain, such as chronic malignant pain.

The present invention will now be illustrated by reference to the following non-limiting examples:

Example 1. Synthesis of a conjugate of NGF and the LH_N fragment of BoNT/A.

Lyophilised murine 2.5 S NGF was dissolved by the addition of water and dialysed into MES buffer (0.1 M MES, 0.1 M sodium chloride, pH 5.0). To this solution (at a concentration of about 0.3 mg/ml) was added PDPH (100 mg/ml in DMF) to a final concentration of 1 mg/ml. After mixing, solid EDAC was

added to produce a final concentration of about 0.2 mg/ml. The reaction was allowed to proceed for at least 30 min at room temperature. Excess PDPH was then removed by desalting over a PD-10 column (Pharmacia) previously equilibrated with MES buffer.

The LH_N fragment of BoNT/A was produced essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* **167**, 175-180. An amount of LH_N equivalent to half the weight of NGF used dissolved in triethanolamine buffer (0.02 M triethanolamine/HCl, 0.1 M sodium chloride, pH 7.8) at a concentration of about 1 mg/ml, was reacted with Traut's reagent (100 mM stock solution in 1 M triethanolamine/HCl, pH 8.0) at a final concentration of 2 mM. After one hour the LH_N was desalted into PBSE (phosphate buffered saline with 1 mM EDTA) using a PD-10 column (Pharmacia). The protein peak from the column eluate was concentrated using a Microcon 50 (Amicon) to a concentration of about 2 mg/ml.

The derivatized NGF was subjected to a final concentration step resulting in a reduction in volume to less than 10 % of the starting volume and then mixed with the derivatized LH_N overnight at room temperature. The products of the reaction were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl-sulphate (SDS-PAGE).

The conjugate resulting from the above reaction was partially purified by size exclusion chromatography over Bio-Gel P-100 (Bio-Rad). The elution profile was followed by measuring the optical density at 280 nm and SDS-PAGE analysis of the fractions. This allowed the separation of conjugate from free NGF and by-products of the reaction.

Fig. 1 shows the SDS-PAGE analysis of the fractions from one such Bio-Gel P-100 column. The free LH_N and conjugate (M_r 100kDa and above) are clearly

separated from the majority of the free NGF (M_r 13kDa). As 2.5S NGF is a homo-dimer formed by non-covalent interactions it is dissociated by treatment with SDS. Thus molecules that have formed covalent cross-links to LH_N through one subunit only will dissociate during the SDS-PAGE analysis and give rise to the free NGF band seen in fractions 4-6. This result demonstrates that the homo-dimeric structure of NGF remains intact after derivatisation. The free LH_N seen in these fractions represents a minor component which has not coupled to NGF. Fractions 4-6 were pooled before further analysis.

Fig. 2 shows an analysis of the conjugate by SDS-PAGE under reducing and non-reducing conditions. Lane 1 is free LH_N under non-reducing conditions, lane 2 is the same amount of LH_N reduced with 50 mM dithiothreitol. Lanes 3 and 4 show the conjugate after size exclusion chromatography either without (lane 3) or with (lane 4) reduction by dithiothreitol. Similarly, lanes 5 and 6 show NGF without or with reduction respectively. The results clearly show that the material in lane 5 with an apparent molecular mass greater than 100kDa produces, upon reduction, the constituent bands of LH_N and NGF only. Furthermore the intensity of the bands following reduction is such that they must be derived from material other than the small amounts of free LH_N and NGF observed in the unreduced sample. The only available source for the excess is the material with an apparent molecular mass >100kDa. The conjugate in the fractions obtained following the size-exclusion chromatography thus represents NGF and LH_N covalently linked by reducible disulphide linkages.

The fractions containing conjugate were stored at 4°C until required.

Example 2. Activities of a conjugate of NGF and LH_N in PC-12 cells.

PC12 cells are a cell-line of neuroectodermal derivation that are commonly used as a model system for the study of nerve function. As a model system for testing the function of a conjugate of NGF and LH_N they have two necessary features: firstly they are well known to possess cell-surface receptors for NGF that have been shown to be involved in a differentiation process in response to low concentrations of NGF. Secondly they have been shown to contain the exocytotic machinery for neurotransmitter release including, importantly in this example, SNAP-25.

PC12 cells were plated out into a 24-well plate that had been coated with MATRIGEL basement membrane matrix (Collaborative Biomedical Products) at a density of approximately 5 x 10^5 cells per well. After a few days in culture (RPMI 1640 with 2 mM glutamine, 10% horse serum and 5% foetal calf serum, 37°C, 5% CO_2) the medium was replaced with fresh medium containing added conjugate (prepared as described in Example 1) or LH_N or no addition. After being kept in culture overnight the medium was removed and the cells washed once with fresh medium. Cells were then lysed by the addition of 0.45 ml sodium hydroxide (0.2 M) for 30 min. After this time the solutions were neutralised by the addition of 0.45 ml hydrochloric acid (0.2 M) followed by 0.1 ml of HEPES/NaOH (1 M, pH 7.4). To extract the membrane proteins from these mixtures Triton-X-114 (10%, v/v) was added and incubated at 4°C for 60 min, the insoluble material was removed by centrifugation and the supernatants were then warmed to 37°C for 30 min. The resulting two phases were separated by centrifugation and the upper phase discarded. The proteins in the lower phase were precipitated with chloroform/methanol for analysis by Western blotting.

The samples were separated by SDS-PAGE and transferred to nitro-cellulose. Proteolysis of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of BoNT/A, was then detected by probing with an antibody that recognises the newly revealed carboxy-terminus of the cleaved SNAP-25 (the antibody is described in Patent Application PCT/GB95/01279). Figure 3 shows an example of such a Western blot. No significant immunoreactivity was observed in samples from control cells (lanes 1 and 2) whereas a band corresponding to a molecular mass of 29 kDa was observed weakly in samples incubated with 10 mg/ml LH_N (lanes 5 and 6) and strongly in samples incubated with 10 mg/ml of the conjugate of NGF and LH_N (lanes 3 and 4). Thus incubation of PC12 cells with the conjugate leads to the marked proteolysis of SNAP-25 indicting that the conjugate has introduced the zinc-dependent proteolytic activity of the L-chain of BoNT/A into the cells' cytoplasm. Little or no such activity was seen with the constituent components of the conjugate.

Incubation of cells with the conjugate in the presence of an excess of free NGF resulted in a reduced production of the proteolytic product of SNAP-25 than did incubation with the conjugate alone. This indicates that the action of the conjugate occurs by means of the NGF targeting moiety interacting with the cell surface receptors for NGF.

Example 3. The activity of a conjugate of NGF and LH_N in primary cultures of dorsal root ganglion neurons.

The dorsal root ganglia contain the cell bodies of primary nociceptive afferents. It is well established that in primary *in vitro* cultures of this tissue the neurons retain many of the characteristics of the nociceptive afferents. These characteristics include the ability to release neuropeptides such as substance P in

response to chemical stimuli known to cause pain *in vivo* (e.g. capsaicin). Furthermore the neurons are known to possess receptors for NGF.

Primary cultures of dorsal root ganglion neurons were established following dissociation of the ganglia dissected from rat embryos (embryological age 12-15 days). The cells were plated into 12 well plates at an initial density of 3×10^5 cells/well in a medium containing NGF (100 ng/ml). After one day in culture fresh medium containing cytosine arabinoside (10 mM) was added to kill non-neuronal cells. The cytosine arabinoside was removed after 2-4 days. After several more days in culture the medium was replaced with fresh medium containing conjugate or LH_N in the absence of NGF. Following overnight incubation at 37°C the medium was removed, the cells were lysed and the hydrophobic proteins extracted using Triton-X-114 as described in Example 2.

The samples were analysed by Western blotting as described in Example 2 with the antibody that recognises the product of the BoNT/A proteolysis of SNAP-25. No immunoreactivity was observed in samples from control cells (lane 4) whereas a band corresponding to a molecular mass of 29kDa was observed weakly in samples incubated with 10 mg/ml LH_N (lane 3) and strongly in samples incubated with 10 mg/ml of the conjugate of NGF and LH_N (lanes 1 and 2).

This result indicates that the conjugate can deliver the proteolytically-active L-chain of BoNT/A into the cytoplasm of the neuronal cells that, *in vivo*, form the primary nociceptive afferents.

Example 4. The production of a chimeric LH_N whereof the L chain is derived from BoNT/B and the H_N fragment from BoNT/A.

The H_N fragment of BoNT/A is produced according to the method described by Shone C.C., Hambleton, P., and Melling, J. (1987, *Eur. J. Biochem.* **167**, 175-180) and the L-chain of BoNT/B according to the method of Sathyamoorthy, V. and DasGupta, B.R. (1985, *J. Biol. Chem.* **260**, 10461-10466). The free cysteine on the H_N fragment of BoNT/A is then derivatised by the addition of a ten-fold molar excess of dipyridyl disulphide followed by incubation at 4°C overnight. The excess dipyridyl disulphide and the thiopyridone by product are then removed by desalting the protein over a PD10 column (Pharmacia) into PBS.

The derivatised H_N is then concentrated to a protein concentration in excess of 1 mg/ml before being mixed with an equimolar portion of L-chain from BoNT/B (>1 mg/ml in PBS). After overnight incubation at room temperature the mixture is separated by size exclusion chromatography over Superose 6 (Pharmacia), and the fractions analysed by SDS-PAGE. The chimeric LH_N is then available for derivatisation to produce a targeted conjugate as described in Example 1.

The examples described above are purely illustrative of the invention. In synthesizing the agent the coupling of the TM to the modified clostridial neurotoxin or fragment thereof is achieved via chemical coupling using reagents and techniques known to those skilled in the art. Thus, although the examples given use exclusively the PDPH/EDAC and Traut's reagent chemistry any other coupling chemistry capable of covalently attaching the TM component of the agent to clostridial neurotoxin derived component and known to those skilled in the art is covered by the scope of this application. Similarly it is evident to those skilled in the art that either the DNA coding for either the entire agent or fragments of the agent could be readily constructed and, when expressed in an appropriate organism, could be used to recombinantly produce the agent or fragments of the agent. Such genetic constructs of the agent of the invention

obtained by techniques known to those skilled in the art are also covered in the scope of this invention.

Table 1- Possible Targeting Moieties (TM)

Growth Factors:

1. Nerve growth factor (NGF);
2. Leukaemia inhibitory factor (LIF);
3. Basic fibroblast growth factor (bFGF);
4. Brain-derived neurotrophic factor (BDNF);
5. Neurotrophin-3 (NT-3);
6. Hydra head activator peptide (HHAP);
7. Transforming growth factor 1 (TGF- 1);
8. Transforming growth factor 2 (TGF- 2);
9. Transforming growth factor (TGF-);
10. Epidermal growth factor (EGF);
11. Ciliary neuro-trophic factor (CNTF).

Cytokines:

1. Tumour necrosis factor (TNF-);

2. Interleukin-1 (IL-1);

3. Interleukin-1 (IL-1);

4. Interleukin-8 (IL-8).

Peptides:

1. -Endorphin;

2. Methionine-enkephalin;

3. D-Ala^2-D-Leu^5-enkephalin;

4. Bradykinin.

Antibodies:

1. Antibodies against the lactoseries carbohydrate epitopes found on the surface of dorsal root ganglion neurons (e.g. monoclonal antibodies 1B2 and LA4);

2. Antibodies against any of the receptors for the ligands given above.

3. Antibodies against the surface expressed antigen Thy1 (e.g. monoclonal antibody MRC OX7).

Claims

1. An agent exhibiting specificity for peripheral sensory afferents which comprises a molecule capable of introducing a protease activity specific for components of the neurosecretory machinery into the cytosol of a primary sensory afferent, and thereby inhibiting the transmission of signals between a primary sensory afferent and a projection neuron by controlling the release of at least one neurotransmitter or neuromodulator from the primary sensory afferent.

2. An agent according to Claim 1 in which the protease activity is the protease activity of the light chain (L-chain) of a clostridial neurotoxin or a fragment thereof retaining the protease activity.

3. An agent according to any preceding Claim which comprises a Targeting Moiety (TM) coupled to a clostridial neurotoxin in which the heavy chain (H-chain) is removed or modified, the TM being capable of functionally interacting with a binding site causing a physical association between the agent and the surface of a primary sensory afferent.

4. An agent according to any preceding Claim which comprises a Targeting Moiety (TM) coupled to a clostridial neurotoxin in which the H_C part of the H-chain is removed or modified.

5. An agent according to Claim 4 in which the modified H-chain is the H_N-fragment of a clostridial neurotoxin.

6. An agent according to any of Claims 2-5 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin.

7. An agent according to any of Claims 2-6 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type A.

8. An agent according to any of Claims 2-6 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type B.

9. An agent according to any of Claims 2-6 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type C_1.

10. An agent according to any of Claims 2-9 in which the H-chain is modified by chemical derivatisation to reduce or remove its native binding affinity for motor neurons.

11. An agent according to any of Claims 2-9 in which the H-chain is modified by mutation to reduce or remove its native binding affinity for motor neurons.

12. An agent according to any of Claims 2-9 in which the H-chain is modified by proteolysis to reduce or remove its native binding affinity for motor neurons.

13. An agent according to Claim 12 which is formed by the coupling of a TM to the LH_N fragment of botulinum neurotoxin type A.

14. An agent according to Claim 12 which is formed by the coupling of a TM to the LH_N fragment of botulinum neurotoxin type B.

15. An agent according to Claim 12 which is formed by the coupling of a TM to the LH_N fragment of botulinum neurotoxin type C_1.

16. An agent according to any of Claims 2-12 in which the H-chain is obtained from a different clostridial neurotoxin than that from which the L-chain is obtained.

17. An agent according to Claim 16 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from botulinum neurotoxin type B.

18. An agent according to Claim 17 which is composed of a TM linked to the H_N fragment of botulinum neurotoxin type A and the L-chain of botulinum neurotoxin type B.

19. An agent according to any of Claims 2-18 in which the L-chain or L-chain fragment is linked to the H-chain by a direct covalent linkage.

20. An agent according to any of Claims 2-18 in which the L-chain or L-chain fragment is linked to the H-chain by a covalent linkage which includes one or more spacer regions.

21. An agent according to any of Claims 2-20 in which the TM is capable of delivering the L-chain or L-chain fragment into the cytosol of a primary sensory afferent unaided.

22. An agent according to any of Claims 2-21 in which the ability to deliver the L-chain or L-chain fragment into the cytosol of a primary sensory afferent is entirely contained within the TM.

23. An agent according to any of Claims 3-22 in which the TM comprises a ligand to a cell-surface binding site on a primary sensory afferent.

24. An agent according to any of Claims 3-23 in which the TM binds to a binding site which is characteristic of a particular defined population of primary sensory afferents.

25. An agent according to any of Claims 3-24 in which the TM binds to a binding site which is characteristic of a particular defined population of primary nociceptive afferents.

26. An agent according to any of Claims 3-25 in which the TM binds to a binding site which undergoes retrograde transport within a primary sensory afferent.

27. An agent according to any of Claims 3-26 in which the TM binds to a binding site which undergoes retrograde transport within a primary nociceptive afferent.

28. An agent according to any of Claims 3-27 in which the TM comprises a ligand to a cell-surface receptor on a primary sensory afferent.

29. An agent according to any of Claims 3-28 in which the TM comprises a ligand to a growth factor receptor on a primary sensory afferent.

30. An agent according to any of Claims 3-28 in which the TM comprises a ligand to a neuropeptide receptor on a primary sensory afferent.

31. An agent according to any of Claims 3-28 in which the TM comprises a ligand to a cytokine receptor on a primary sensory afferent.

32. An agent according to any of Claims 3-28 in which the TM comprises a ligand to a hormone receptor on a primary sensory afferent.

33. An agent according to any of Claim 3-32 in which the TM comprises a monoclonal antibody or is derived from a monoclonal antibody to a surface antigen on a primary sensory afferent.

34. An agent according to Claim 29 in which the TM comprises a ligand to a nerve growth factor receptor.

35. An agent according to Claim 34 in which the TM comprises nerve growth factor.

36. An agent according to Claim 35 which comprises nerve growth factor linked to the LH_N fragment of botulinum neurotoxin type A.

37. An agent according to any preceding Claim in which the TM is linked to the clostridial neurotoxin-derived component by a direct covalent linkage.

38. An agent according to any preceding Claim in which the TM is linked to the clostridial neurotoxin-derived component by a covalent linkage which includes one or more spacer regions.

39. An agent according to any preceding Claim which prevents the release of a neurotransmitter or neuromodulator from a primary sensory afferent.

40. An agent according to any preceding Claim which inhibits the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent.

41. A method for obtaining an agent according to any preceding Claim which comprises the covalent attachment of a TM to a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin.

42. A method for obtaining an agent according to any of Claims 1-40 which comprises the covalent attachment of a TM to a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin with the inclusion of one or more spacer regions.

43. A method for obtaining an agent according to any of Claims 1-40 which comprises constructing a genetic construct which codes for a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin , incorporating said construct into a host organism, expressing the construct to produce the modified clostridial neurotoxin or fragment of a clostridial neurotoxin and the covalent attachment of the modified clostridial neurotoxin or fragment of a clostridial neurotoxin to a TM.

44. A method for obtaining an agent according to any of Claims 1-40 which comprises constructing a genetic construct which codes for a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin , incorporating said construct into a host organism, expressing the construct to produce the modified clostridial neurotoxin or fragment of a clostridial neurotoxin and the covalent attachment of the modified clostridial neurotoxin or fragment of a clostridial neurotoxin to a TM with the inclusion of one or more spacer regions.

45. A method for obtaining an agent according to any of Claims 1-40 which comprises constructing a genetic construct which codes for the agent, incorporating said construct into a host organism and expressing the construct to produce the agent.

46. A method of controlling the release of a neurotransmitter or neuromodulator from a primary sensory afferent by applying the agent of any one of Claims 1-40.

47. A method of controlling the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent by applying the agent of any one of Claims 1-40.

48. A method of controlling the transmission of sensory information from a primary sensory afferent to a projection neuron by applying the agent of any one of Claims 1-40.

49. A method of controlling the transmission of sensory information from a primary nociceptive afferent to a projection neuron by applying the agent of any one of Claims 1-40.

50. A method of controlling the sensation of pain by applying the agent of any one of Claims 1-40.

51. Use of the agent according to any one of Claims 1-40 or a pharmaceutically acceptable salt thereof as a medicament for the alleviation of pain.

52. Use of the agent according to any one of Claims 1-40 or a pharmaceutically acceptable salt thereof as a medicament for the prevention of pain.

53. Use of the agent according to any one of Claims 1-40 in the manufacture of a medicament for the alleviation of pain.

54. Use of the agent according to any one of Claims 1-40 in the manufacture of a medicament for the prevention of pain.

55. A method of alleviating pain which comprises administering an effective dose of the agent according to any one of Claims 1-40.

56. A method of preventing pain which comprises administering an effective dose of the agent according to any one of Claims 1-40.

57. An agent exhibiting specificity for peripheral sensory afferents which can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents, the agent having the following discreet functions:

1) It binds to a surface structure (the Binding Site [BS]) which is characteristic of, and has a degree of specificity for, nociceptive afferent neurons;

2) It enters the neuron;

3) It enters the cytosol; and

4) It modifies components of the exocytotic machinery present in the synaptic terminals of the central processes of the neurons, such that the release of at least one neurotransmitter or neuromodulator from the synaptic terminal is reduced or prevented.

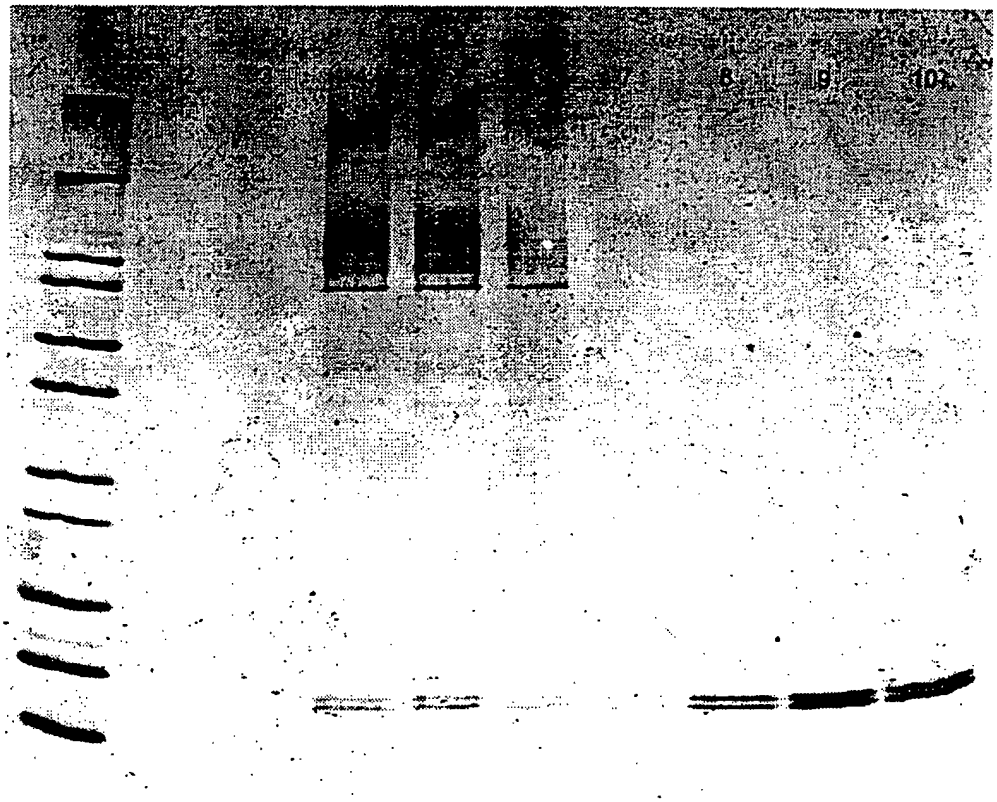

Figure 1

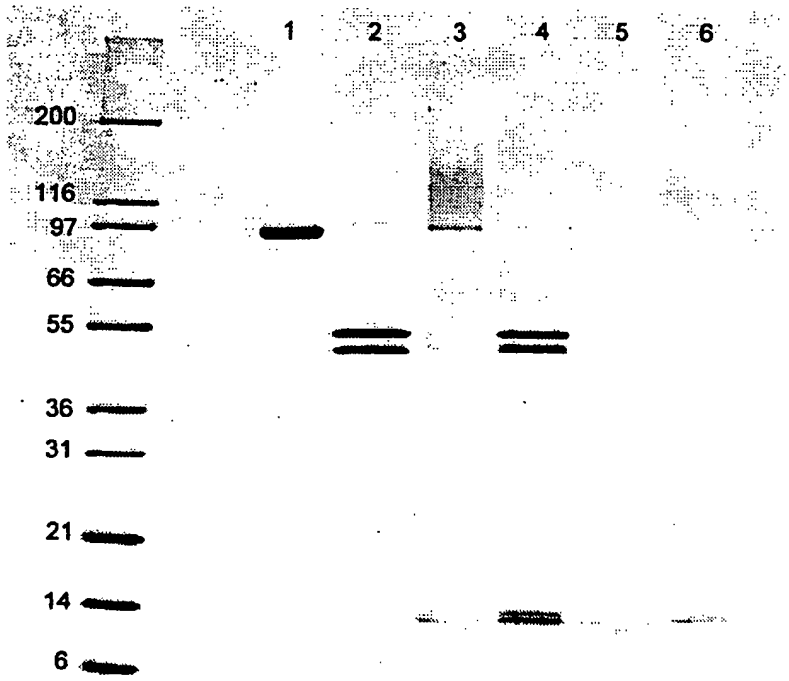

Figure 2

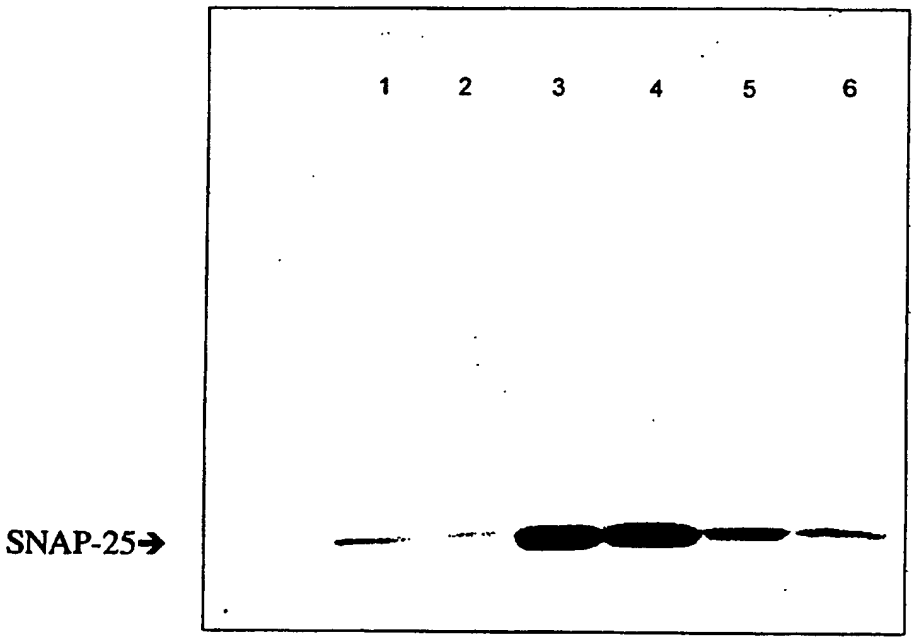

Figure 3

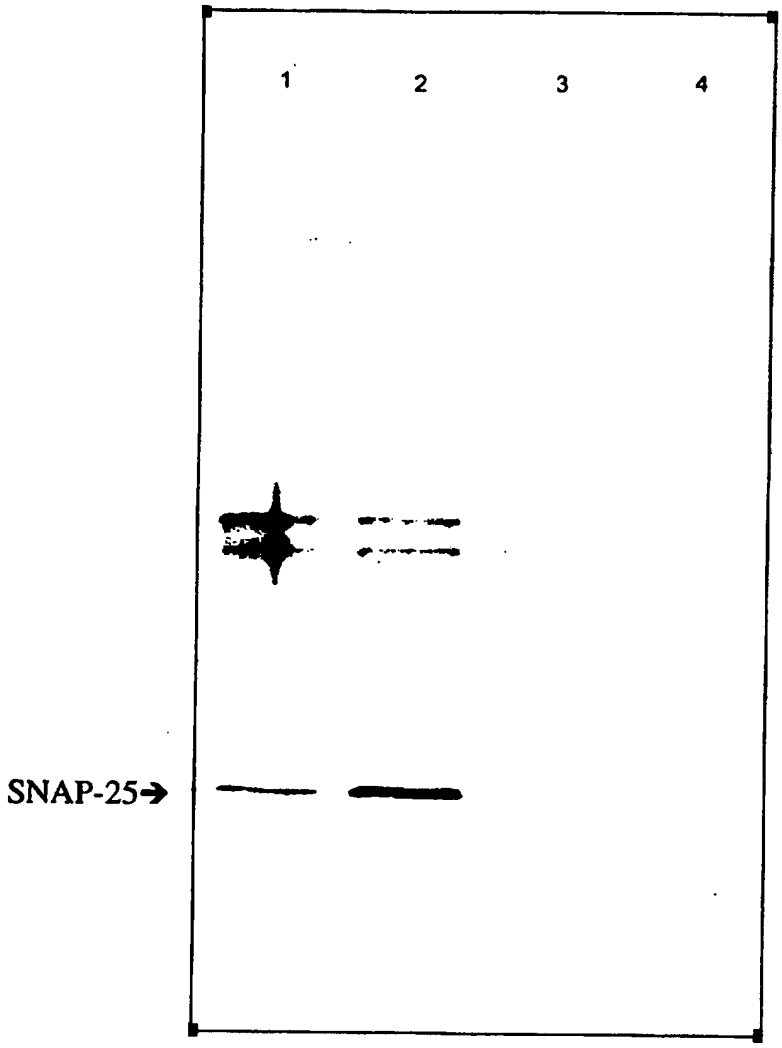

Figure 4

INTERNATIONAL SEARCH REPORT

Interr ·nal Application No
PCT/GB 96/00916

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K19/00 C07K14/33 C12N9/52 C07K14/48
C07K14/475 C07K14/52 C07K14/575 C07K16/18 C12N15/62
A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,94 15629 (ASS SYNAPSE BIOLOG) 21 July 1994 see the whole document ---	1-15, 19-57
Y	WO,A,94 28923 (ALLERGAN INC) 22 December 1994 see the whole document ---	1-15, 19-57
Y	JOURNAL OF NEUROLOGY, vol. 239, 1992, pages 16-20, XP002009346 P. HAMBLETON: "Clostridium botulinum toxins: a general review of involvement in disease, structure, and mode of action and preparation for clinical use" see the whole document --- -/--	1-15, 19-57

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 1996	01.08.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Van der Schaal, C

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 129 434 (UNIV TEXAS) 27 December 1984 see the whole document ---	1-15, 19-57
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 33, 25 November 1992, MD US, pages 23479-23483, XP002009347 G. SCHIAVO ET AL: "Botulinum neurotoxons are zinc proteins" see page 23479, left-hand column, last line - right-hand column; figure 1 ---	2-15, 19-56
Y	MEDLINE DATABASE Accession Nr. 9563023 XP002009349 & HOKKAIDO JOURNAL OF MEDICAL SCIENCE, vol. 70, no. 1, January 1995, pages 19-28, N. FUJII: "Structure and function of botulinum toxin" see abstract ---	2-15, 19-56
Y	MEDLINE DATABASE Accession Nr. 96018139 XP002009350 & TOXICON, vol. 33, no. 4, April 1995, pages 559-567, F. LEBEDA AND M. OLSON: "Structural predictions of the channel-forming region of botulinum heavy chain" see abstract ---	2-15, 19-56
Y	BIOLOGICAL ABSTRACTS, vol. 97, Philadelphia, PA, US; abstract no. 225145, POULAIN B: "Molecular mechanism of action of tetanus toxin and botulinum neurotoxins." XP002009351 see abstract & PATHOLOGIE BIOLOGIE 42 (2). 1994. 173-182. ISSN: 0369-8114, ---	2-15, 19-56
Y	WO,A,95 01806 (KONDRATYEV ALEXEI) 19 January 1995 see the whole document ---	21-32, 34-56
Y	EP,A,0 544 292 (BOEHRINGER MANNHEIM GMBH) 2 June 1993 see the whole document --- -/--	21-32, 34-56

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 96/00916

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 16, 1990, MD US, pages 9153-9158, XP002009348 T. BINZ ET AL: "The complete sequence of botulinum neurotoxin type A..." see the whole document	43-45

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

I· ··rnational application No.
PCT/GB96/00916

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 46-52, 55 and 56
because they relate to subject matter not required to be searched by this Authority, namely:
Although these claims are directed to a method of treatment of the human/ animal body the search has been carried out and based on the alleged effects of the compound/composition

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/00916

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9415629	21-07-94	AU-B-	6030494	15-08-94
		CA-A-	2153781	21-07-94
		EP-A-	0679090	02-11-95
WO-A-9428923	22-12-94	AU-B-	7101894	03-01-95
		EP-A-	0702559	27-03-96
EP-A-0129434	27-12-84	US-A-	4664911	12-05-87
		AU-B-	570049	03-03-88
		AU-B-	2948084	03-01-85
		CA-A-	1232838	16-02-88
		GB-A,B	2142032	09-01-85
		JP-B-	6039387	25-05-94
		JP-A-	60069033	19-04-85
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		AU-B-	7320394	06-02-95
		EP-A-	0712313	22-05-96
EP-A-0544292	02-06-93	DE-A-	4139001	03-06-93
		JP-A-	6303987	01-11-94